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Variation in Egg Size and Number in Drosophila subobscura

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VARIATION IN EGG SIZE AND NUMBER IN DROSOPHILA SUBOBSCURA

Maternal and paternal effects revealed through
intrapopulation and interpopulation crosses

A Thesis

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Minyoung Janet Yi

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This thesis is submitted in partial fulfillment of

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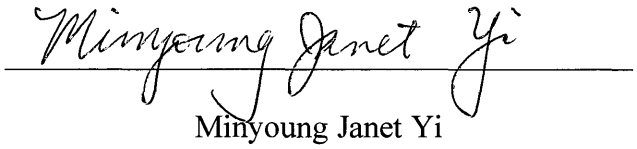

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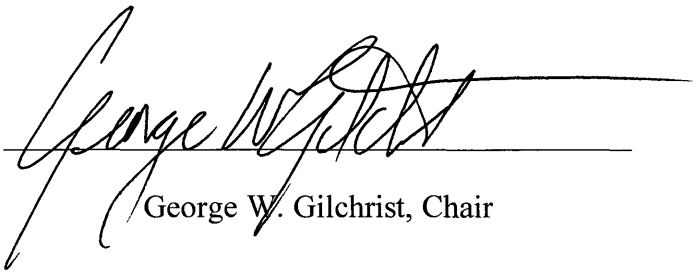
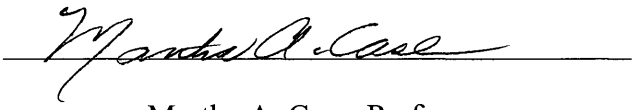
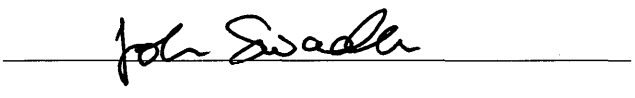


George W. Gilchrist, Chair



Martha A. Case, Professor



John P. Swaddle, Professor

My parents who imbued in me an appreciation for learning.

Materi Ecclesiae who taught me all the things worth knowing.

Mariae semper virgini who points me closer to the Truth every day.

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ABSTRACT

Drosophila subobscura was recently introduced into North America and has since evolved clinal variation in morphological characters. This variation resembles the patterns seen in ancestral European populations. We studied whether the reproductive characters of egg size and egg number also exhibited clinal patterns that were similar between the two continents. We measured and counted eggs for the first 5-6 days of laying from high and low latitude populations for each continent. We predicted that high latitude populations would have larger eggs that might be attributable. In Europe, northern flies had bigger eggs. In North America, southern flies had bigger eggs. For egg number, we predicted that high latitude populations would have higher early life fecundity than low latitude populations. In *D. melanogaster* latitude is inversely correlated with egg-to-adult development time; if development time is a proxy for sexual maturation then high latitude populations should lay more eggs sooner. In Europe, we found that early life fecundity was higher in the northern population than in the southern population. We found no such difference in North America. The egg size and number differences between the continents suggest that a simple temperature-based explanation does not suffice. Competition and desiccation tolerance may be operating in the introduced populations.

We also performed a series of interpopulation crosses and tested for changes in egg size and number relative to the parental population. Although previous interpopulation cross studies have found male × female interactions on fecundity, no one has yet found an interaction on egg size. This is partly because egg size is assumed to be under maternal control in oviparous taxa. However, we found significant male effects and male × female interactions on egg size. This is an unusual result for a species like *D. subobscura*, wherein the eggs are fertilized immediately prior to oviposition and hatch shortly after oviposition. These effects maybe due to behavioral or biochemical interactions between the sexes. Because of the fitness consequences of egg size, male × female interactions on egg size may influence post-zygotic reproductive isolation or be an avenue for males to precipitate sexual conflict.

VARIATION IN EGG SIZE AND NUMBER IN DROSOPHILA SUBOBSCURA

CHAPTER I

LITERATURE REVIEW

Interpopulation crosses: the ugly, the bad, and the good

What can the outcomes of interpopulation crosses reveal about the processes or mechanisms that determine male-female coevolutionary trajectories? Researchers have proposed two interpretations of the results of interpopulation crosses based upon a scenario of male-female coevolution. The first states that interpopulation crosses can reveal signatures of sexually antagonistic coevolution (SAC) (Andrés and Arnqvist 2001). If females have coevolved with males from their own population via sexual conflict, the sexes will have different optima for their reproductive traits, such as re-mating rate. As a result, males and females may have to constantly circumvent each other's manipulations to maintain their own optimum. This conflict over re-mating occurs because male reproductive success is generally dependent upon the number of mates he can copulate with (or the number of copulations), but this is not the case with females (Bateman 1948). In fact, excessive copulations or courtship harassment can even cause harm to females (i.e. Carayon 1966). Under a scenario of SAC, female hormone receptors may evolve mechanisms for depressing the hormonal stimulations to re-mate with a co-evolved male (homopopulation or intrapopulation male). These males keep up the chase by evolving mechanisms to stimulate females more successfully. However, if a female is exposed to a male from a different population (heteropopulation or interpopulation male), her receptors may be less efficient at binding to his hormones. She

may not be able to depress his novel hormonal signals. Thus, interpopulation males may be able to induce females to re-mate more often, beyond the optimum desirable for female fitness. Foreign males may elicit a higher re-mating rate with interpopulation females, relative to intrapopulation females. This sort of release from the female inhibition has been observed for various traits in interpopulation crosses. Examples of released inhibition in insects include: higher re-mating rates and oviposition rates (Andrés and Arnqvist 2001), higher initial female reproductive rate and increased life-time offspring production (Nilsson et al. 2002), and increased fertilization success in sperm competition (Wilson et al. 1997; Clark et al. 1999; Hosken et al. 2002). These results have been interpreted to mean that SAC is operating in the original populations from which individuals were taken.

Conversely, a second interpretation suggests that interpopulation crosses may reveal or reaffirm the presence of incipient reproductive isolation via female choice. If females have coevolved with their own males via female mate choice, they will respond best to males from their own population (or species) and discriminate against allopatric males. The coevolved intrapopulation males will have higher re-mating rates, increased fertilization success, or higher offspring production, relative to interpopulation males. In other words, different populations may have diverged significantly enough to have form weak reproductive barriers within the species. Knowles and Markow (2001) found that interpopulation crosses of desert *Drosophila* produced larger insemination reaction masses in the female reproductive tract; reaction masses are large opaque vaginal accumulations that occur after mating and prevent oviposition until the mass has subsided. Brown and Eady (2001) demonstrated that sympatric males out-performed

allopatric males in terms of sperm competition and oviposition stimulation in the beetle species *Callosobruchus maculatus*. Rivera et al. (2004) found that males from allopatric populations of the damselfly *Calopteryx haemorrhoidalis* differed in their mode of sperm removal. Sperm removal increases male fertilization and is an important component of sexual selection in damselflies. The occurrence of different modes of sperm removal in different populations suggested incompatibility between interpopulation males and females. Such results indicate that weak reproductive barriers can form among populations within a species. If this is the case, sexual conflict within a population cannot be detected via interpopulation crosses, as females will always respond best to their own males with whom they have coevolved via sexual selection.

To distinguish between SAC and female choice that results in reproductive isolation, theoretical modeling has taken up this problem and questioned the diagnostic utility of interpopulation crosses. Rowe et al. (2003) outlined a model of male-female conflict over mating rate in different theoretical strains. They developed a single signal-receptor model; there is a single female receptor that determines re-mating rate and a single male signal that stimulates re-mating. They altered two parameters – the female threshold (i.e. how much male stimulation is required) and female sensitivity to male stimulation (i.e. how the female responds) – and then calculated male × female interactions among strains. The existence of a male × female strain interaction meant that different female strains had different responses when presented with the same male. Interactions did not occur for a single signal-receptor system when only female threshold was allowed to evolve. Interactions, however, did occur for a single-receptor system when both threshold and sensitivity were allowed to evolve. In these models, females

had the highest mating rate with their own males. This result was contrary to the empirical results and verbal arguments presented by Andrés and Arnqvist (2001) and suggested that interpopulation crosses cannot alone diagnose SAC. Re-examination of previous studies (Nilsson et al. 2002; Nilsson et al. 2003) and additional empirical work (Long et al. 2006) have substantiated some of Rowe et al.'s (2003) conclusions that the female responses in interpopulation crosses are greater than, similar to (Hebets and Maddison 2005), or do not vary in a consistent direction from, the female responses in intrapopulation crosses (Wilson et al. 1997; Fricke and Arnqvist 2004; Long et al. 2006).

Furthermore, although Rowe et al. (2003) assumed sexual conflict, they reasoned that when female sensitivity is allowed to evolve, Fisherian or good-genes assumptions could also yield male × female interactions. After all, there may be female choice for genetically different males to obtain indirect genetic benefits for her offspring. This may be to avoid inbreeding as interpreted by Attia and Tregenza (2004).

Despite the apparent difficulty in assessing SAC through interpopulation crosses, it is nonetheless true that strong male × female interactions have been observed empirically when mating individuals from different populations or strains (Wilson et al. 1997; Clark et al. 1999; Andrés and Arnqvist 2001; Nilsson et al. 2002; Hosken et al. 2002; Hebets and Maddison 2005). Thus, interpopulation crosses can detect variation triggered by mating interactions. Even if we have no idea as to how these interactions are occurring, the salient point stands: we would never arrive at the "how?" if first we did not know that male × female interactions were occurring. Interpopulation crosses that result in strong male × female interactions are therefore at least a starting point to study SAC and female choice. Once the existence of interactions is established, follow-up studies

can try to determine the mechanism (i.e. female choice, drift, sexual conflict, genetic incompatibilities).

Furthermore, although Rowe et al.'s (2003) theoretical work suggested that interpopulation crosses cannot diagnose SAC, this was true for only a single signal-receptor system. It is still unclear if the diagnostic ability of interpopulation crosses for SAC will improve for a multiple signal-receptor model. Rowe et al. (2003) address this concern but they maintain that the basic results of their model will not change in a more complex scenario. While Rowe et al.'s results remain illuminating, further elaboration of a more complex signal-receptor system is required since most male-female mating interactions are under the simultaneous control of multiple signal-receptors (e.g. the Acp system in *Drosophila*).

The degree of population differentiation is another potentially confounding factor in interpreting interpopulation crosses (Chapman et al. 2003; Rowe et al. 2003; Arnqvist and Rowe 2005). In particular, as populations diverge over time, female response to interpopulation male traits will decline due to reproductive isolation (Arnqvist and Rowe 2005). This may partially explain the presence of weak reproductive barriers in desert *Drosophila*, the bruchid beetle, and damselflies. Initially, females under SAC are predicted to respond most strongly to closely related allopatric males, but this effect disappears as genetic divergence increases (Arnqvist and Rowe 2005). One measure of population differentiation is $F_{ST,\ which}$ is the amount of heterozygosity in a certain population divided by the total heterozygosity across all populations. However, F_{ST} can differ based on the loci examined; for mating interaction studies, F_{ST} should be based upon loci that code for hormones or receptors involved in mating rather than upon neutral

markers. Selection appears to be strong on at least male hormones that are transferred during copulation (i.e. Acp system in *Drosophila*). Therefore F_{ST} may appear to be highest with respect to Acp loci. When differentiation is near complete ($F_{ST} \approx 1$), females should perform the best with their own males. When there is almost no population differentiation ($F_{ST} \approx 0$), females should perform the same across all male types (since, technically, it is one big population). Chapman et al. (2003) proposed that researchers should consider population differentiation when interpreting the results of interpopulation crosses.

With these considerations in mind, I performed a series of interpopulation crosses using *Drosophila subobscura* from different latitudes and continents. I measured the reproductive traits of fecundity and egg size and analyzed whether the crosses differed with respect to the parental populations. Although interpopulation crosses as a diagnostic tool for revealing process has been dismissed (Tregenza et al. 2006), they nonetheless offer a way to detect variation emerging from mating interactions. They can still reveal underappreciated phenomena, validating their continued use. Observations of oviposition rates, sperm precedence, and re-mating rates made through interpopulation crosses that show variation among mating trials can be a red flag. Such findings can reveal systems where conducting more deductive tests for sexual conflict or incipient reproductive isolation might prove fruitful. In my study, I believe that, interpopulation crosses can be useful to test whether or not egg size and fecundity of a particular female will change depending on the population origin male partner.

The sexual conflict over progeny size

Parental investment can be defined as "anything done by the parents for the offspring that increases the offspring's chance of surviving while decreasing the parent's ability to invest in other offspring" (Trivers 1974). From this definition, it is clear parents and offspring will conflict over the amount of parental investment allocated to progeny. One such conflict can occur over the size of their common offspring. Parents desire to optimize both progeny size and number to maximize their reproductive success. To accomplish this (in the simplest case), parents may invest equally across all their offspring. Investing too little or too much into different offspring will not maximize overall reproductive success. On the other hand, progeny will attempt to garner as many resources (or care behaviors) that increase their own fitness. An individual will always be more related to itself than it will be to its siblings and its parents, provoking selfish actions and culminating in parent-offspring conflict.

This conflict over parental investment has recently been analyzed as a male-female battle, in addition to a parent-offspring one. Male and female conflict over the optimal size of their common offspring exists in angiosperm plants (Haig and Westoby 1989) and mammals (Moore and Haig 1991). This conflict may have led to the evolution of genomic imprinting, which is a parent-sex-specific epigenetic mark that causes the expression of only one allele of a gene in the progeny. In mice, the maternal copy of the growth promoter *Igf2* is silenced, which enables only the paternal copy to function (DeChiara et al. 1991). This monoallelic expression in the embryo induces the female to provide more nutrients to the embryo, despite the risk of lowering her own future reproductive success. This makes sense evolutionarily for polygamous species like mice

where males desire only to ensure the success of their own offspring – not those that might be sired by other males with the same female in the future. Genomic imprinting appears to be unique to organisms in which the embryo is dependent upon the mother for nutrition during development. It is absent in oviparous species like insects, presumably because egg size is determined before fertilization and because the embryo persists outside of the mother after fertilization.

The conflict over progeny size is also interesting because it has been proposed as a postzygotic reproductive isolation mechanism for organisms like plants and mice (Kondoh and Higashi 2000). Females do not sit idly by while their progeny's *Igf2* gene expression is monoallelic. The corresponding receptor for *Igf2*, *Igf2r*, is also imprinted; *Igf2r* tags *Igf2* for destruction making *Igf2r* in effect a growth suppressor (Haig and Graham 1991). Growth suppression may prevent the female from investing superfluously into one offspring, thereby saving some resources for other present or future progeny. Kondoh and Higashi (2000), in their model, investigated the potential for a coevolutionary "arms race" between growth suppressor and promoters. They concluded that if this race occurs, mismatch between species for suppressors and promoters may lead to abnormal progeny development. In effect, this mismatch would be a postzygotic reproductive isolation mechanism among species, preventing the formation of hybrids.

Although genomic imprinting of growth promoters and suppressors does not occur in oviparous species (O'Neill et al. 2000), an analogous phenomenon may. Sexual conflict over progeny size may exist in insects like *Drosophila*. It is already known that male flies can stimulate increased rates of oogenesis and oviposition. Might males have

other means of affecting the reproductive investment responses of females? Even though the *Drosophila* embryo has very little opportunity to manipulate maternal provisioning since eggs are laid immediately after fertilization, paternal effects on egg size have been known to occur in crickets (Weigensberg et al. 1998) and salmonids (Pakkasmaa et al. 2002). I investigate the possibility for a male effect on egg size in *D. subobscura*.

Conducting interpopulation crosses in D. subobscura

I crossed geographically disparate populations of *D. subobscura* to characterize the female response to intrapopulation males versus interpopulation males, with respect to the reproductive characters of egg size and egg number. Because the direction and strength of the response that females have in interpopulation crosses have been found to be erratic, both in empirical tests and theoretical models, I refrain from making any directional predictions about the magnitude of the female response. Rather, the goals of this study were two-fold. Firstly, I assessed population variation for egg size and egg number to obtain a baseline measurement. Secondly, I tested if interpopulation crosses produce eggs that differ in size and number from the intrapopulation crosses. I looked to see if interpopulation males can elicit smaller or larger progeny with respect to intrapopulation males.

D. subobscura is an attractive system in which to conduct our study for two reasons. Firstly, *D. subobscura*'s population history and population genetics have been well studied. Measures of differentiation (F_{ST}) are available for the populations used in this study (Pascual et al. 2001), which enables us to make inferences about the effect of population differentiation on reproductive characters in interpopulation crosses. Secondly, latitudinal and continental differences in morphology have been studied

extensively in this species, while latitudinal differences in fecundity have been studied in a close relative, *Drosophila melanogaster*. As a result, we are able to take into account possible historical or adaptive reasons for why interpopulation crosses may not vary in regard to egg size or egg number. I discuss our system in more detail below.

Population history and population genetics of D. subobscura.

D. subobscura is a Palearctic fruit fly in the *obscura* species group of the *Drosophila* clade. Its native range distribution is from North Africa to the southern regions of Scandinavia (Krimbas 1993). In 1978 *D. subobscura* was discovered in Puerto Montt, Chile, and has since become a common drosophilid throughout South America from 29° S to 53° S (Budnik and Brncic 1982; Prevosti et al. 1987; Ayala et al. 1989). In 1982 it was also discovered in Port Townsend, Washington, and has become prevalent along the western coast of the United States with a range from 34° N to 50° N (Prevosti 1988; Krimbas 1993). Because the New World populations have been so well documented and studied since the time of their introduction, the *D. subobscura* system has offered a unique opportunity to study the pace and trajectory of evolution. Hailed as a "grand experiment in evolution" (Ayala et al. 1989), the New World populations have demonstrated that the pace of evolution can be remarkably fast as well as repeatable, albeit with minor but discernable differences (Huey et al. 2000).

Analyses have revealed that both North and South American populations share the same chromosomal inversion polymorphisms (Beckenbach and Prevosti 1986; Prevosti et al. 1988; Ayala et al. 1989; Balanya et al. 2003), lethal genes (Mestres et al. 1990; Mestres et al. 1992), *rp49* haplotypes (Rozas and Aguade 1991), and allozyme alleles (Balanya et al. 1994). These data support the hypothesis that both New World

colonizations represent genetic replicates from the same European source population and not independent introductions from different locales. Additionally, these genetic analyses suggest that the New World founding populations was relatively small, as only a subset of genetic variation found in Europe appeared in the New World. The New World populations possess only 18 chromosomal polymorphisms whereas there are >80 different existing European inversions; furthermore, they do not possess any of the more rare inversions (Prevosti et al. 1988). Only 8 of 70 *rp49* haplotypes were present in the New World populations (Rozas and Aguade 1991). Rarer allozyme alleles are also absent in the New World (Balanya et al. 1994).

More recent work on microsatellite variation and inferences from Approximate Bayesian Computation (ABC) have identified the original source population of the invasions to be the western Mediterranean region of Europe, particularly Barcelona, Spain (Pascual et al. *in press*). The ABC method has shown strong support for an initial *D. subobscura* introduction with founder effects from Europe into South America, followed by a serial introduction from South America into North America. ABC posterior distributions have proposed also that the South American population was established by ~ 7 effective founders, while the North American population was established by 100-150 effective founders (Pascual et al. *in press*).

Depending on the genetic markers used, the *D. subobscura* populations within a continent show varying degrees of population differentiation. For example, microsatellite work has shown that there is very little differentiation within Europe and North America. However, populations that are located farther apart seem to have slightly higher F_{ST} values (Pascual et al. 2001). Based on these values, the *D. subobscura* populations

within the continents appear to experience a great deal of gene flow. The number of migrants per generation (Nm) in Europe is observed to range between 3 and 83; in North America Nm = 2 – 40 (Pascual et al. 2001).

Despite high gene flow within the continents, detailed studies of chromosomal inversion polymorphisms have revealed extensive genetic structure within the continental populations. *D. subobscura* has five pairs of acrocentric chromosomes, which are all polymorphic for chromosomal inversions. The frequencies of these inversions demonstrate clinal variation along latitudes in the ancestral European populations (Prevosti 1964; Krimbas 1993). Amazingly, less than a decade after the New World introductions, the chromosomal inversion frequencies in the New World had also achieved clines that were often in the same direction as the Old World populations (Prevosti et al. 1988; Prevosti et al. 1990). Subsequent surveys have confirmed that although the slopes of the New World inversion clines were not of the same magnitude as those in Europe, they were still present two decades following the introductions (Balanya et al. 2003). Comparisons of regions within the O_{ST} and O_{3+4} chromosomal arrangements have revealed that different arrangements can possess significantly different genetic structure (Munté et al. 2005).

Geographical variation in morphological characters in D. subobscura

Researchers have suggested that sufficient additive genetic variation is necessary for populations to respond to environmental change (Holt et al. 2003; Lee 2002). The strong bottleneck and founder events in the New World populations have not reduced genetic diversity enough to constrain *D. subobscura*'s ability to adapt to a new environment and subsequently to spread across a large latitudinal range. Work on clinal

variation in the New World populations has revealed that *D. subobscura* females from both North and South American populations have evolved wing size clines in just under two decades that parallel European clines (Huey et al. 2000; Gilchrist et al. 2001; Gilchrist et al. 2004). Female flies from high latitudes have larger wings than flies from low latitudes. At a rate of size divergence at ~0.011 haldanes (~1700 darwins), this is one of the highest rates of evolution measured for a quantitative trait, leading to the conclusion that natural selection in the New World populations is extremely strong. The remarkable repeatability of clines in geographically disparate regions and their constancy over time supports the action of natural selection rather than genetic drift, migration, or other evolutionary forces (Endler 1986). Larger wing size at higher latitudes may represent an adaptation to cooler temperatures that decrease wing loading (Gilchrist and Huey 2004).

Despite the extreme interest in the *D. subobscura* colonization events as a "grand experiment in evolution", no one has yet studied variation in fecundity or egg size among the continents. Because the New World populations have responded morphologically to latitudinal and temperature gradients, it is probable that the phenotypic characters of egg size and fecundity in *D. subobscura* will likewise demonstrate clinal variation, as long as there is sufficient additive genetic variation with respect to these traits. Egg size is positively correlated with aspects of larval fitness (discussed later). If *D. subobscura* has survived in the New World at different latitudes, it may have evolved local adaptations with regard to egg size. For example, it has already been shown that egg size varies clinally in *D. melanogaster* (Azevedo et al. 1996). High latitude flies have larger eggs than flies from lower latitudes. These differences may be temperature mediated, as

laboratory experiments have shown that egg size has a plastic response to oviposition and rearing temperatures (Azevedo et al. 1996; Crill et al. 1996; Azevedo et al. 1997). These egg size differences may also be adaptive (Fischer et al. 2005a). Hence I predict that higher latitude flies will have larger eggs than lower latitude flies. I tested this prediction by measuring egg size in high and low latitude population flies from Europe and North America.

It is as yet unclear if there is clinal variation for fecundity or fecundity profiles in *Drosophila melanogaster*. Laboratory experiments suggest that cool-adapted flies may differ in fecundity patterns from warm-adapted flies. Partridge et al. (1995) found that cool adapted flies had earlier fecundity peaks than warm adapted flies when both lines were tested at the same temperature. This difference may be attributable to the fact that both laboratory cold-adapted lines and flies from northern latitudes have slightly faster developmental rates than warm-adapted or southern latitude flies (Partridge et al 1994; James and Partridge 1995). However, it is as yet unclear if cold-adapted flies reach their fecundity peak sooner because they are able to initiate egg laying sooner.

If there is latitudinal variation for fecundity peaks due to temperature, one might expect *D. subobscura* to vary in a similar manner. However, in the present study I did not conduct a long-term observation of fecundity profiles. Rather, I investigated fecundity over a shorter period of time – the first 5-6 days of egg laying. With such a short observation period, it would be impossible to assess whether northern and southern flies differed in fecundity profiles. Instead, I observed whether the flies differed in their egg production during this early period. If development time can be taken as a proxy for sexual maturation time, I expect that high latitude populations will lay more eggs early on

than low latitude populations. Northern flies will have higher initial fecundity as might be expected for a cold-adapted fly.

What will interpopulation crosses reveal in D. subobscura*?*

Given the information available about the population history and genetic differentiation in *D. subobscura*, as well as additional work on clinal variation in a close relative, *D. melanogaster*, it is clear that the *D. subobscura* system is an attractive one in which to conduct interpopulation studies. As stated earlier, using *D. subobscura* will enable us to determine the importance of genetic differentiation, on the degree of the female response to interpopulation males. In addition, I will be able to assess whether egg size or egg number change depending on the source population of the male. If egg size can change due to paternal effects, this would encourage further investigations into possible mechanisms. Other studies have observed that egg number can change and there is some indication that these changes are mediated through biochemical interactions between the sexes.

Trade-offs between egg size and egg number

Life history is very broadly defined as the study of the relationship between traits that affect reproduction and survival, including size at birth, lifespan, and age of maturation (Stearns 1992). While, in general, large egg size is beneficial in many respects, egg size is subject to trade-offs with other aspects of fitness as predicted by life history theory. A desire to study the trade-off between progeny size and number has stimulated a wealth of models (Smith and Fretwell 1974; Parker and Begon 1986; Winkler and Wallin 1987; Lloyd 1987; Sakai and Harada 2004). Smith and Fretwell (1974) provided the first mathematical formulation of the optimization of progeny size

that continues to be used by biologists. They made two basic assumptions: a) increased parental investment per offspring (I_{young}) increases progeny fitness (W_{young}) and b) the number of progeny (N) depends upon the total amount of parental investment (I_{Total}), which is a fixed value:

$$N = \frac{I_{Total}}{I_{young}} \quad (1)$$

Hence, parental fitness is a function of progeny number and the fitness of the young:

$$W_{Parent} = N \; x \; W_{young} \quad (2)$$

From this simple relationship, we can see clearly that progeny size and progeny number are inversely correlated. In general, this pattern is observed in semelparous arthropods (Ebert 1993) and the trend is also present after correction for body size in iteroparous arthropods (Carriere et al. 1995), but there are exceptions. Previous work in *D. subobscura* has not identified a trade-off (Avelar and Pite 1989). However, this study did not statistically correct for body size. I predict a trade-off between egg size and number in *D. subobscura* will occur after a body size correction. In a study by Berrigan (1991) a taxonomic-wide trade-off was present after a correction for body mass in the Order Diptera (which included a data point from *D. subobscura*). His results suggest that a trade-off should be present within *D. subobscura.*

When a size-number trade-off is not evident within a species, this is probably due to two reasons. Firstly, Smith and Fretwell's (1974) assumption of fixed resources may be unrealistic for many organisms. Fox and Czesak (2000) suggested that additional theoretical work is required to understand the optimization of egg size (and number) when resource availability or an individual's ability to allocate resources to reproduction

varies. The relationship between resource acquisition and allocation on life-history trade-offs has been mathematically explored (van Noordwijk and de Jong 1986). Van Noordwijk and de Jong's model explained that when variation among individuals for acquisition ability exceeds variation in allocation ability, positive correlations between life history traits arise. In other words, if some individuals are better at acquiring food than others, they can invest these resources into all aspects of life history, leading to a positive correlation among variables. Such models have not yet been applied to the egg size vs. egg number scenario, although empirically, this seems to be the case (Avelar and Pite 1989; Steigenga et al. 2005).

Secondly, a size-number trade-off may not occur because there are other factors that have not been considered. After all, an individual must allocate resources to three basic tasks – growth, somatic maintenance, and reproduction. It is probable that trade-offs are occurring at these other levels, which were not examined (Fox and Czesak 2000). Charlesworth (1990) demonstrated this idea in the context of quantitative genetics: although some life history traits are expected to correlate negatively, their relationships with other unmeasured traits can result cause positive correlations between the variables of interest. Instead, the traits of interest may have negative correlations with the unmeasured variables. I did not assess other life-history traits to assess the potential for trade-offs at other levels.

Egg production in Drosophila *flies*

Before reviewing the sources of egg size and egg number variation in flies, a brief discussion of the biology of egg production is required. Detailed studies of oogenesis have been conducted for *D. melanogaster* (King 1970). These studies should be largely

applicable to *D. subobscura* as it is similar to *D. melanogaster* in some aspects of reproductive biology. Two species groupings are recognized based on differences in oocyte maturation relative to the life cycle (Mahowald and Kambysellis 1980). The first category includes species like *D. melanogaster* in which the egg follicles form in the pupal stage and remain previtellogenic (i.e. no yolk) until adult eclosion. Vitellogenesis, or yolk formation, occurs shortly after eclosion. In *D. melanogaster*, copulation, and oviposition occur within a day after eclosion. The second category includes species like the Hawaiian *Drosophila* in which the egg follicles form during the adult phase of the life cycle. In such species, vitellogenesis occurs 15-20 days after eclosion. In contrast to *D. melanogaster*, *D. subobscura* begins egg laying a couple of days after eclosion (personal observation). Although *D. subobscura* is different from *D. melanogaster* in many aspects of its breeding ecology, it is still not as dramatically different as the Hawaiian *Drosophila*. Ovaries, although smaller with respect to body size compared to *D. melanogaster*, are already present in the *D. subobscura* female after eclosion (Atkinson 1979). Hence, *D. subobscura* is more appropriately placed in the first category of species, so we can apply observations in *D. melanogaster* to *D. subobscura.*

Eggs in *D. melanogaster* are produced continuously and not simultaneously. The oocytes undergo various stages of development, numbered 1 thru 14, in the ovaries of virgin females (Figure 1) (King 1970). Ovaries contain multiple ovarioles, which are "independent egg assembly lines" that produce new oocytes and contain continuously maturing eggs (King 1970). Vitellogenesis occurs immediately after eclosion in oocytes in the developmental stages 8 and 10 (Bownes 1986). As the virgin female matures, the ovaries gradually accumulate mature stage 14 eggs that are ready for oviposition.

FIGURE 1

ANATOMY OF DROSOPHILA MELANOGSTER FEMALE REPRODUCTIVE TRACT IN DORSAL AND LATERAL PERSPECTIVES

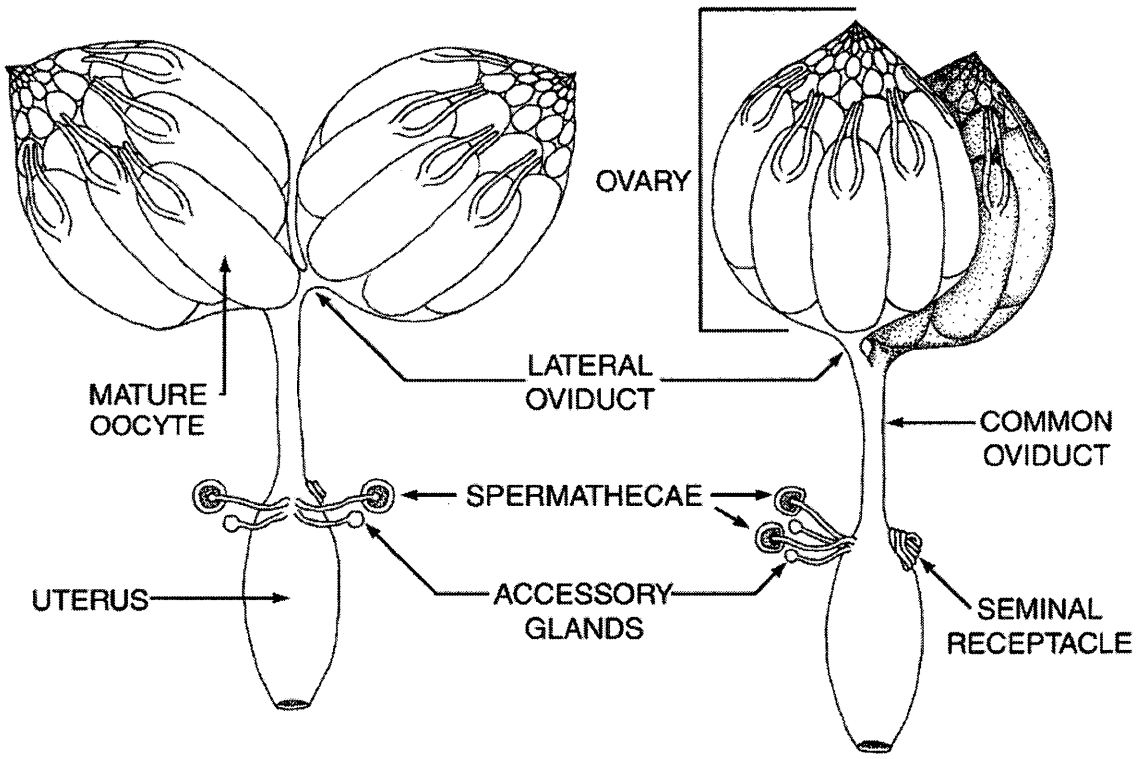


Figure drawn by Jacqueline Grant (from Bloch Qazi et al. 2003).

However, in virgins, oogenensis eventually ceases and oocytes in developmental stage 9 or before undergo resorption. After the female mates, oogenesis resumes, and the additional oocytes progress towards stage 14. Component of the male ejaculate, stimulates oogenesis and continued egg maturation, allowing yolk uptake to occur during stage 10 (Soller et al. 1999). I discuss the implications of this coordinated regulation for interactions between the sexes below.

The causes of variation in egg size

Egg size is a fundamental life history trait with profound fitness consequences in a wide variety of taxa including fish, birds, reptiles, and insects (see review in Azevedo et al. 1997). In *Drosophila melanogaster*, egg size is positively correlated with hatching success, hatchling weight, and larval feeding rate (Azevedo et al. 1997). In other species, egg size is also positively correlated with pre-adult size, and often with adult size as well (Azevedo et al. 1997). In turn, large body size can affect additional components of adult fitness. For example, in *D. subobscura* it is known that larger males that produce larger regurgitation droplets for females have a mating advantage over smaller males (Steele 1986). In female flies, large size is positively correlated with fecundity (Parsons 1964; Berrigan 1991; Reeve and Fairbairn 1998).

In *Drosophila melanogaster*, egg size seems to have an underlying genetic basis. At least one autosomal gene and one sex linked gene affect egg size (Warren 1924). Laboratory experiments have demonstrated that egg size is a heritable trait (Azevedo et al. 1997) and that it responds to selection regimes for increased and decreased lengths (Parsons 1964; Schwarzkopf et al. 1999). The latitudinal clines in egg size that have been documented in *D. melanogaster* likely result from these genetically based

differences (Azevedo et al. 1996; Azevedo et al. 1997). Studies have shown that additivity is a large component of the genetic variance for egg length (Mazumadar and Prabhu 1968). There is some suggestion that egg size in *D. melanogaster* may be under stabilizing selection (Curtsinger 1976a; Curtsinger 1976b) but other studies suggest directional selection (Roff 1976).

There are also non-genetic factors, such as maternal inheritance, that influence egg size in insects. Maternal inheritance is broadly defined as the influence of the maternal phenotype and environment upon the fitness of the offspring. Maternal inheritance can affect egg size in addition to genetic predispositions for size. Maternal decisions and factors that influence provisioning into eggs include oviposition substrate (Fox et al. 1997), environmental temperatures (Azevedo et al. 1996, Fischer et al. 2003a), and diet (Fox 1993). Cytoplasmic effects are a prominent example of maternal inheritance; the egg cell confers all or most of the organelles, RNA transcripts, and cytoplasm to the embryo when compared to the smaller sperm cell (Davidson 1986). These transcripts and organelles affect the phenotype of the progeny. For example, it is known that nurse cells in the ovaries deposit mRNA transcripts in the oocyte; these transcripts eventually format the anterior-posterior axis in the embryo (reviewed in Gilbert 2006). Axis patterning involves a strong maternal inheritance component. Any significant differences between the reciprocal hybrid lines indicate strong maternal or cytoplasmic effects, beyond purely Mendelian inheritance (Carson and Lande 1984). In the present study, all the population crosses were conducted under the same environmental and rearing conditions. Thus, any differences in population hybrids must be due to either cytoplasmic effects (i.e. populations vary in some component of yolk or

RNA deposition), or to differences in female responses to different male types (i.e. males of different populations stimulate differences in yolk deposition).

In addition to genetic and maternal sources of variation, egg size in insects has a plastic component that responds to an array of environmental factors (for review see Fox and Czesak 2000). Egg size plasticity due to oviposition and rearing temperatures has been documented extensively for a variety of species such as butterflies (Fischer et al. 2003c), flies (Avelar 1993; Azevedo et al. 1996; Crill et al. 1996; Blanckenhorn 2000), and beetles (Ernsting and Isaaks 1997). In general, insects lay larger eggs when raised or ovipositing at lower temperatures. This phenomenon has been attributed to either a) physiological limitations or benefits caused by temperature (Richards 1964; Bradford 1990) or b) an evolutionary adaptive response caused by trade-offs between size and survivability in different temperatures (Yampolsky and Scheiner 1996; Fischer et al. 2003a; Fischer et al. 2003b). Alternatively, egg size plasticity in response to temperature may itself be adaptive (Fox and Czesak 2000). For instance, genetic variation for a plastic response to temperature in egg size has been found in the butterfly *Bicyclus anynana*, suggesting that egg size plasticity may itself be adaptive (Steigenga et al. 2005).

Mindful of the temperature-mediated plasticity in egg size, I chose to assess differences in egg size among *D. subobscura* populations reared in a common garden. By rearing these populations at the same temperature for multiple generations (~60), I have standardized the environment and am thus able to assess differences in egg size due to genetics (and cytoplasmic effects). Standardizing the environment for multiple generations also excludes the possibility of cross generational effects due to population differences from the field. I propose that *D. subobscura* demonstrates a genetic basis for

egg size differences among high and low latitude populations. These differences are probably due to selection for large and small eggs in the high and low latitude environments respectively, caused by an adaptive advantage, physiological constraint, or perhaps selection on a correlated trait.

In addition, I paired female flies with males from different continental or latitudinal populations. The manipulation of crosses may be considered a change in the environment, since males from different populations may elicit different responses in egg size, either through behavioral or a biochemical interactions with the female. Elicitation of different egg sizes, *per se*, due to interactions between the sexes has not been documented previously in *Drosophila*. Azevedo et al. (1997) examined crosses between *Drosophila melanogaster* lines in which sexually mature females (aged 5-7 days at 25°C, or 9-11 days at 18° C) had small and large eggs. They found that egg size was completely dependent upon the genotype of the mother. Large-egg mothers produced large eggs even if they are mated to males with the small egg genotype. Work in butterflies has also shown that male rearing temperature has no effect on the female partner's egg size (Fischer et al. 2003). These authors argue that it is unlikely that males can affect egg size in females because egg size is determined prior to fertilization. This is a reasonable conclusion, but since they conducted observations over a short period of time, their methods might not have been sensitive enough to detect male-effects. In contrast, I measured eggs from the day of first day of laying until the flies were aged 8 days. Thus I am able to obtain repeated measurements as well as to discover whether there is any time dependent effect. Collecting data at different time points may prove to

be a more sensitive approach, which may allow me to uncover subtle male-effect differences.

Furthermore, male-mediated effects on size have been shown to occur in other insects. In the cricket *Gryllus firmus*, male genetics and investment were shown to affect egg size. In *G. firmus*, initial egg size is under the control of the female. However, eggs take several days to mature after oviposition and during this time they undergo size changes due to metabolism and water uptake; this study found an effect of male genotype on size in these older eggs (Weigensberg et al. 1998). However, no one has yet found an effect of male type on egg size in insects like *Drosophila*, for which egg size does not change appreciably after oviposition. In *D. melanogaster* male rearing temperature has been shown to affect egg size in daughters (Crill et al. 1996), although the mechanism for such a cross-generational correlation is unclear. Nonetheless, the peculiar effects of male genotype in *G. firmus* and male rearing temperature in *D. melanogaster* on egg size leads us to ask whether this is a more general effect: does exposure to different male types elicit differences in egg size within the same generation? While it is clear that egg size is under genetic control by the female, its plastic response to environmental variables suggests that plastic changes may occur in response to different male types. If this is the case, then male influences on egg size are potentially under appreciated for taxa like *D. melanogaster* in which fertilization and oviposition occur in immediate sequence.

The causes of variation in fecundity

Fecundity, or egg number (a.k.a. clutch size), is another fundamental life history trait with profound fitness consequences. Studying egg number complements our analysis of egg size variation and provides a more complete life history picture of *D.*

subobscura (discussed further below). As with egg size, the sources of variation in fecundity can be classified into genetic, maternal, and environmental. I discuss these sources and then discuss their relevance to *D. subobscura.*

Robertson (1957) concluded that 60% of the variance in egg production is due to genetic variation in *D. melanogaster*. Fecundity has significant but low heritability (Tait and Prabhu 1970), as might be expected for a trait that is important to fitness (Roff and Mousseau 1987). Selection typically reduces the additive genetic variance, which decreases heritability (V_A / V_{Total}). Selection upon fecundity resulted in lower egg number in the downward selection regime; in the upward regime, egg number increased but not significantly (Reeve and Fairbairn 1998). This suggests that although the genetic variance for fecundity has not been depleted, it has been reduced. Observations of fecundity profiles and development time in *D. melanogaster* have shown that cold-adapted and warm-adapted flies are different. As explained earlier, cold-adapted flies experience early life fecundity, while southern populations demonstrate higher late life fecundity (Partridge et al. 1995). Other studies have demonstrated that southern populations mature more slowly from egg to adult than northern population (James and Partridge 1995). It is as yet unclear if individuals from southern populations reproduce earlier as a consequence of taking longer to mature.

The observation of differences in clutch size elicits the question: why is there variation? The literature regarding clutch size evolution can be distilled into comparisons of short-term versus long-term strategies (Price 1997). Lack was the first to address clutch size variation. He hypothesized that clutch size is determined by natural selection; parents produce only as many offspring as for which they can provision. Lack saw this

as the primary explanation for intraspecific latitudinal differences in clutch size in birds: "Most birds breed in summer, and so experience a longer day the further they are from the tropics. A longer day will, in general, enable parent birds to collect more food each day, and so will enable them to feed larger families at one time" (Lack 1947). Lack's hypothesis is a density-dependence based argument that considers fine-tuning in real time by an organism. A second hypothesis that explains differences in fecundity is the balanced mortality hypothesis (Price 1974), which states that egg production is related to the hostility of the environment. Animals such as parasites have high fecundity (i.e. *r*, the intrinsic rate of increase, is high) when the chances of mortality are high. This argument is invoked when considering evolution of the species in the long term. Although there is clinal variation in fecundity for insects (Peschken 1972; Mitrovski and Hoffmann 2001; Schmidt et al. 2005a; Schmidt et al. 2005b), the applicability of Lack's hypothesis or the balanced-mortality hypothesis is unclear.

Nonetheless, clutch size in *D. subobscura* has important fitness consequences. Like many other *Drosophila* species, *D. subobscura* will oviposit on rotting fruit (although the major breeding substrates are unknown) (Atkinson 1979). *D. subobscura* lays eggs on these fruits in large aggregations (Atkinson and Shorrocks 1984) that experience Allee effects, whereby survivability is lowest at the lowest densities (Rohlfs and Hoffmeister 2003). No one has yet tested mechanisms to explain why *D. subobscura* egg-to-adult survival is greatest at intermediate densities. In the jack-pine sawfly, larvae work cooperatively to break and feed on the epidermis of the plant (Ghent 1960). It is possible that such mechanisms operate in *D. subobscura* clutches. Wertheim et al. (2002) and Rohlfs (2005) demonstrated that *Drosophila melanogaster* larvae aggregate on

fungal-infected patches, which may be a defensive behavior to break up fungal filaments that otherwise would increase larvae mortality. Hence, aggregation of eggs and larvae may be adaptations to increase fitness.

Additional factors that determine clutch size have been examined in other insects (Godfray 1987; Godfray et al. 1991). In these discussions, the Lack clutch size (which is different from Lack's hypothesis) is the clutch size that maximizes parental fitness. This requires optimizing the relationship between clutch size and the per capita fitness of the individuals in the clutch. In other words, parental fitness is not highest for the largest clutches, due to increased offspring competition, predation, or parasitism for larger clutches. Furthermore, trade-offs with future reproduction probably explain why the observed clutch sizes are usually smaller than the Lack clutch size in insects (Godfray 1987).

In *D. melanogaster*, fecundity is also sensitive to maternal effects and environmental effects experienced by both parents. For example, it is clear that egg number is positively correlated with female body size (Robertson 1957). However, female oviposition temperature has a large effect on fecundity; females ovipositing at 25°C produce more eggs than females ovipositing at 18° C (Huey et al. 1995). Egg production also varies with female age (Rose 1984): egg numbers gradually increase until it peaks and then numbers decrease with age. Fecundity is strongly influenced by environmental factors such as nutrition (Robertson 1957), crowding (Robertson and Sang 1944), and even male presence or density (Hoffmann and Harshman 1985).

Fecundity has also been shown to vary depending upon the genotype of the male partner. Andrés and Arnqvist (2001) performed a series of reciprocal mating experiments

among genetically differentiated houseflies, *Musca domestica*. In their study, they observed that females had the highest oviposition and remating rates when mated to males from different populations. They conclude that this result is consistent with antagonistic co-evolution of the sexes. Females are able to mitigate the effects of seminal fluid proteins of a male with whom she has co-evolved; by contrast, foreign males would be able to by-pass the female's receptor system and stimulate oviposition rates. Although such studies have suggested that strong interpopulation interactions are consistent with antagonistic co-evolution between the sexes, the good-genes hypothesis, or even out-breeding vigor, might also explain the same results (Rowe et al. 2003, Arnqvist and Rowe 2005; Attia and Tregenza 2004). Nonetheless, male effect and male-female interaction effects on egg number is intriguing. How and why is this occurring? Although the results from interpopulation crosses may be inconclusive, many of the results beg the question of mechanism. For example, it is interesting to note that while we know interpopulation crosses result in different egg number relative to intrapopulation crosses, and that copulation stimulates oogenesis and oviposition, we do not yet know if these two are causally related. Researchers have identified Acps that cause egg laying, but have not demonstrated if amino acid substitutions (that presumably vary among populations) in these proteins result in decreased or increased binding affinity to female receptors. If this causation were established, there would be more empirical support for Andrés and Arnqvist's (2001) original assertion that interpopulation differences can reveal signatures of SAC.

Sexual conflict in the Drosophila *system*

Although an isolated virgin fly synthesize new eggs, the physical presence of the male (Hoffmann and Harshman 1985) and copulation itself stimulate an increased rate of oogenesis and ovulation (Chen et al. 1988; Heifetz et al. 2000; Saudan et al. 2002). Over 80 Acps are transferred to the female during copulation and are involved in male-female antagonistic interactions (Wolfner 2002). Acp70, or sex peptide, stimulates *de novo* oogenesis (Chen et al. 1988). Acp26Aa, ovulin, stimulates ovulation – or the release of mature oocytes (Heifetz et al. 2005). Furthermore, because fertilization first requires proper sperm storage, the first eggs oviposited by a female are fertilized at a lower rate than eggs produced later on. In addition, recent work has shown that Acps are embedded in the egg shell. Their role in the shell is uncertain although they are suspected to be anti-microbial agents (Ram and Wolfner 2005).

Since male hormones partially initiate the signals for vitellogenesis, they may also subsequently affect egg size. Although males can stimulate (or manipulate) oogenesis or oviposition rates (Holland and Rice 1999; Andrés and Arnqvist 2001), no one has yet looked for manipulation in vitellogenesis rates. It is already suspected that the amount of yolk deposited in eggs is sensitive to temperature (Ernsting and Isaaks 1997). But it is unclear whether males can affect yolk uptake by oocytes inside of the female. Studying the ability to manipulate vitellogenesis rates (or oocyte time spent in vitellogenesis) would represent a next step in understanding how males can influence egg size and open up inquiries into sexual conflict over progeny size in oviparous taxa. A recent study has shown that three Acps enter the ovary (Ram and Wolfner 2005). These Acps have not yet been functionally characterized, but their target in the ovaries leaves one to ponder

whether or not these proteins are capable of influencing vitellogenesis rates. If my study can demonstrate the existence of a male effect on size, then it is quite possible that this effect is mediated through Acps found in the ejaculate. However, there are other explanations, including differential allocation by the female (Burley 1986). According to the differential allocation hypothesis, females invest more into reproduction given the attractiveness of their male partners. Females may exhibit their preference for certain male types by allocating more yolk into eggs fertilized by attractive males. Although this theory has been tested in birds for reproductive success (de Lope and Møller 1993; Swaddle 1996), adequate testing remains to be done in insects. Another behavioral mediated effect on egg size may also occur; females may experience differential energy expenditure rates due to differences in courtship harassment rates by interpopulation males. There may also be differences in embryonic genetic expression that affect egg size; however, because *Drosophila* eggs do not change appreciably in size between oviposition and hatching, genetic effects in this regard may be less important.

In any event, enhancing our understanding of male effects on female reproductive investment decisions is very worthwhile. Although the interpopulation cross technique has been called into question as a diagnostic tool for SAC, its merits are still manifold. This technique may yet uncover unusual or unexpected mating interactions that may suggest where additional investigation might prove valuable. Such pioneering studies will improve our knowledge of what avenues are available for males to manipulate in precipitating sexual conflict, or what means females have at their disposal to make cryptic decisions in favor of, or against, the hapless contributor of a haploid complement of chromosomes.

CHAPTER II

INTRODUCTION

Paternal contributions to egg size in oviparous species such as insects have long been underappreciated. It is often assumed that egg size is almost exclusively pre-determined by the mother. This is not an unreasonable assumption, as yolk uptake and oocyte maturation occur largely prior to fertilization in the reproductive tract of females. Thus it seems that males can only affect egg size through their daughters; male genetic contributions to egg size are only seen in the grandchildren (Reznick 1981). However, Weigensberg et al. (1998) found paternal genetic and paternal environmental effects on cricket egg size at 10 days after oviposition. Work in salmonid fishes showed that paternal contributions on final egg size occurred after fertilization, during the swelling phase (Pakkasmaa et al. 2002). It is conceivable that final pre-hatch egg size can be mediated by male genetic contributions; males may contribute alleles that enable progeny to metabolize or absorb nutrients from the environment at differential rates. No study has yet considered the effect that males may have on egg size in species where significant changes in egg size do not occur prior to hatching. While this seems an unlikely area for a male to intervene, it clear that males do play active roles that affect the reproductive investment decisions of their female mates. Males can transfer resources through their ejaculates (i.e. Markow et al. 1990; Karlsson 1998), through externally proffered nuptial gifts (i.e. Thornhill 1976; Steele 1986; Cumming 1994), or both to the female. These resources provide nutrition for the female and/or become incorporated into making eggs

(see reviews Vahed 1998; Arnqvist and Nilsson 2000). Males can also transfer ejaculate hormones that stimulate oogenesis, ovulation, and oviposition (Chen et al. 1988; Heifetz et al. 2000; Saudan et al. 2002). Most studies have quantified the effect that male nutrition and hormones have on female reproductive investment in terms of fecundity, or egg number. A few studies have examined the relative contribution of resources to egg number versus egg size (Wedell and Karlsson 2003).

With the recent explosion of interest in sexual conflict, it makes sense to test carefully the assumption that egg size is wholly under maternal supervision. For example, male and female conflict over the optimal size of their common offspring exists in plants (Haig and Westoby 1989) and mammals (Moore and Haig 1991). This conflict may have caused the evolution of genomic imprinting. A genomic imprint is a parent-sex-specific epigenetic mark that causes the expression of only one allele of a gene in the progeny. In mice, the maternal copy of the growth promoter *Igf2* is silenced, which enables only the paternal copy to function (DeChiara et al. 1991). This monoallelic expression in the embryo induces the female to provide more nutrients to the embryo, despite the risk of lowering her own future reproductive success. This makes sense evolutionarily for polygamous species where males desire only to ensure the success of their own offspring – not those that might be sired by other males with the same female in the future. Genomic imprinting appears to be unique to organisms in which the embryo is dependent upon the mother for nutrition during its development. As such, it is absent in oviparous species like insects, presumably because egg size is determined before fertilization and because the egg is released immediately after fertilization.

Conflict over progeny size may yet exist in insects like *Drosophila,* even though the embryo has very little opportunity to manipulate maternal provisioning. Trivers (1974) pointed out that we might expect to see parent-offspring disagreement over parental investment in sexually reproducing organisms in general. And especially because egg size in insects (and other oviparous animals), is correlated with components of fitness (Azevedo et al. 1997; Fox and Czesak 2000), the progeny have a stake in their initial size. Large egg size is positively correlated with increased hatching success, juvenile survival, desiccation resistance, and starvation resistance (Fox and Czesak 2000). Egg size is also positively correlated with pre-adult size, and often with adult size as well (Azevedo et al. 1997). In turn, large body size can affect adult fitness (Steele 1986; Reeve and Fairbairn 1998). Although large egg size is beneficial in many respects, egg size is subject to environmentally induced plasticity and trade-offs with other aspects of fitness. In insects, egg size is particularly responsive to rearing and oviposition temperature.

Trade-offs between egg size and number are found to occur in insects such as flies, beetles, and butterflies (reviewed in Fox and Czesak 2000). The study of this trade-off has stimulated a wealth of models (Smith and Fretwell 1974; Parker and Begon 1986; Winkler and Wallin 1987; Lloyd 1987; Sakai and Harada 2004). However, trade-off models fail to consider the conflicts that males and females of polygamous insect species may experience over egg size. Because large egg size is so beneficial and because it appears to trade-off with number of progeny, it is possible that male and female insects have different optima for the size of their common progeny. A female maximizes her reproductive success by provisioning enough to each of her offspring, without investing

superfluously; thereby she reaches some optimization of both size and number of offspring. On the other hand, as in mammals, an insect male may desire the eggs he fertilizes with any females to be large, despite the future fitness cost incurred by the female partner for too-large eggs. We may then ask: is it possible for a male to influence egg size when there appears to be so much maternal control in species like *Drosophila*?

In an effort to address this question, we performed a series of interpopulation crosses in *Drosophila subobscura*. The *D. subobscura* system is an attractive system in which to conduct studies of egg size differences for three reasons. Firstly, *D. subobscura*'s population history and genetic structure have been well studied. *D. subobscura* is native to the Palearctic region but it was recently introduced into North and South America. Inferences from Approximate Bayesian Computation (ABC) have identified the original source population of the New World invasions to be the western Mediterranean region of Europe, particularly Barcelona, Spain (Pascual et al. *in press*). There are also measures of population differentiation (F_{ST}) available for the populations used in this study (Table 1; Pascual et al. 2001). This enables us to assess the importance of population differentiation in interpreting the results of interpopulation crosses. Secondly, latitudinal and continental differences in morphology have extensively studied in this species (Huey et al. 2000; Gilchrist et al. 2001; Gilchrist et al. 2004), while latitudinal and temperature mediated differences in reproductive characters have been studied in a close relative, *Drosophila melanogaster* (Partridge et al. 1994; James and Partridge 1995; Azevedo et al. 1996; Schmidt 2005a; Schmidt 2005b). As a result, we are able to take into account possible historical or adaptive reasons for why interpopulation crosses may not vary in regard to egg size or egg number. All previous

TABLE 1

GENETIC DIFFERENTIATION (F_{ST}) BETWEEN DROSOPHILA SUBOBSCURA POPULATIONS

	Aarhus	Barcelona	Bellingham	Fort Bragg
Aarhus	-	0.007	0.108	0.098
Barcelona		-	0.103	0.095
Bellingham			-	0.006
Fort Bragg *				-

These F_{ST} values are calculated from microsatellite loci and are re-published from Pascual et al. (2001).

* Fort Bragg, CA (39° 29' N, 123° 43' W) is located 180 miles west of Davis, CA. Microsatellite variation from the Fort Bragg *D. subobscura* population was used as a proxy for the Davis *D. subobscura* population.

studies of interpopulation crosses to study sexual conflict have not considered the effects geographical variation may impose on reproductive characters. This variation may constrain the response that females can have to interpopulation males. Thirdly, gene orthologues of *D. melanogaster* accessory gland proteins (Acps) are present in *D. subobscura* (as well as in other *Drosophila* flies) (i.e. Cirera and Aguade 1998; Mueller et al. 2005). A male fly, upon mating with a female fly, transfers sperm and 80-100 Acps produced in the male accessory gland organ. Some Acps have been shown to increase male fitness at the female's expense. Furthermore, Acps have undergone rapid protein evolution, suggesting an arms race between male and female reproductive molecules: females are actively circumventing manipulation while males continue to try new things.

Are males from different populations capable of changing the size of the eggs that females lay, relative to the eggs she lays when mated to more familiar males? Or: are females capable of changing her own egg size depending on her partner? We propose to answer this question using the technique of interpopulation crosses. The technique of interpopulation crosses to unambiguously assess sexual conflict has recently come into question (Rowe et al. 2003; Tregenza et al. 2006). Theoretical and empirical observations suggest that patterns of increased oviposition rates or re-mating rates in interpopulation crosses relative to intrapopulation crosses are not indicative of the process of sexual conflict alone. Similar patterns may occur by other modes of sexual selection, or even by natural selection, drift or gene flow. Moreover, the patterns produced by interpopulation crosses in models and in empirical trials are often inconsistent (i.e. increased, decreased, or the same female responses relative to intrapopulation crosses) making their interpretation difficult. However, in the rush to

discredit this technique to diagnose sexual conflict, its great virtue has been overlooked: the ability it offers to detect variation triggered by mating interactions. Thus, while we acknowledge that the results of interpopulation crosses cannot indicate the action of sexual conflict, and that the results of such crosses can be erratic, they can still reveal underappreciated phenomena, validating their continued use. Here we report our finding that both males and females affected egg size in an oviparous insect species. We found that populations varied in egg size. These population differences may be the result of selection by abiotic factors such as temperature and aridity or by biotic factors such as interspecific competition. There was a strong maternal effect on egg size; overall egg size was in broad terms consistent among all eggs laid by a certain population female, regardless of the origin of her mate. This effect likely reflects a genetic pre-disposition for size. Nonetheless, our data show decisively that males from different populations were able to change egg size to some degree. These changes did not show any correlation with the degree of population differentiation or the geographical origin of the individuals in the cross. Rather, initial egg size was the only factor that determined changes in egg size. Although the paternal influence is a smaller effect than the maternal influence, we conclude that male effects upon egg size should not be ignored. Considerations of the male and female conflict over progeny versus progeny number may not be confined to viviparous taxa. We discuss possible mechanisms for this "male-effect" modification and implications of these findings.

CHAPTER III

MATERIALS AND METHODS

The *D. subobscura* populations Bellingham (BE) and Davis (DA) were collected from North America in April 2004. The Aarhus (AA) and Barcelona (BA) populations were collected from Europe in May 2003. All populations were sampled during the spring breeding seasons and with the same field collection methods. BE and AA comprise the northern populations used in this study, while DA and BA comprise the southern populations (Figure 1 and Table 1). Between 15 and 25 isofemale lines were established from the wild for each locale. The lines were reared on standard molasses-cornmeal medium and maintained at 20°C on a 14 hr light: 10 hr dark cycle. After two generations, 10 males and 10 females were taken from each isofemale line and combined into population cages (25 cm x 14 cm x 12 cm). The population cages were maintained for over one year prior to this study. To obtain flies for the crosses conducted in this study, we put in bottles with fresh molasses-cornmeal medium into the population cages and collected eggs the next day. Fifty eggs were transferred into each of 21 vials and reared to adulthood for each population.

Intra-continental and inter-continental crosses

In order to study differences in egg size among continental populations, we crossed flies from among the four populations. Crosses were set up between 1 day old flies and were performed in 6 rounds to obtain all possible combinations of matings (Figure 2 and Table 2). The hybrid eggs from reciprocal crosses allowed us to observe

TABLE 2

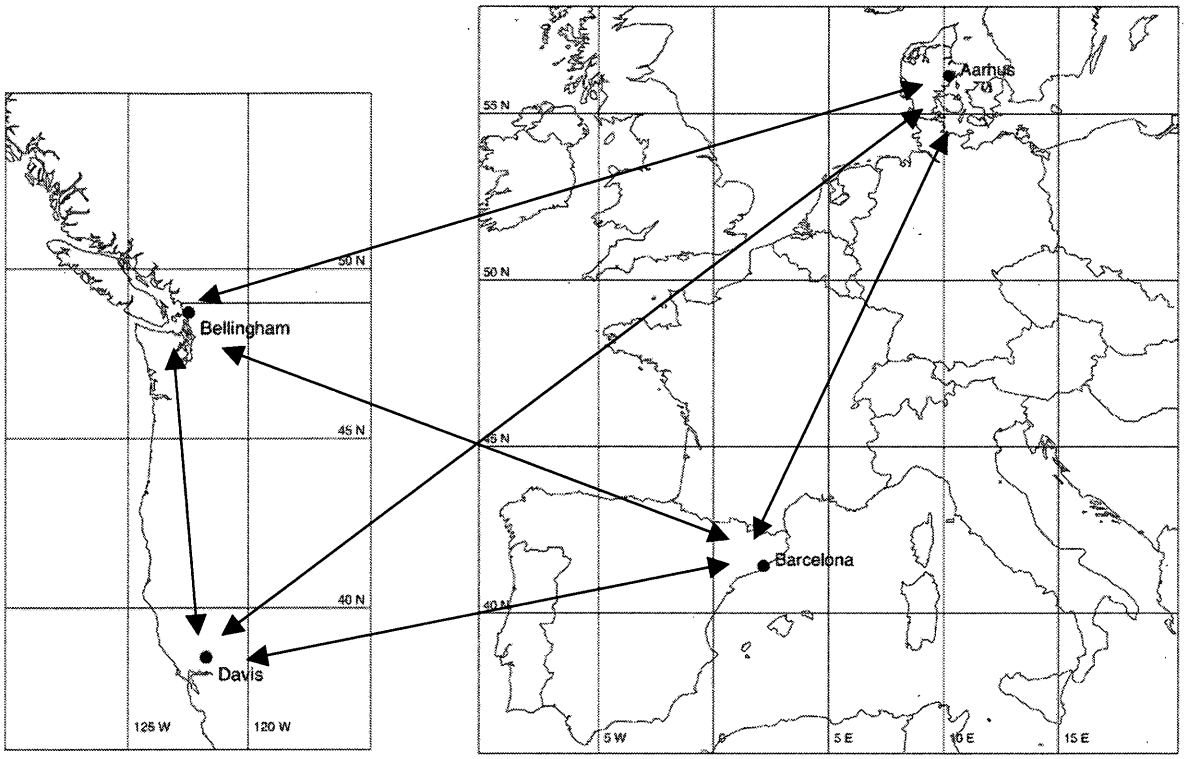
DROSOPHILA SUBOBSCURA POPULATIONS

Continent	Location	Population	Abbreviation	Latitude	Longitude
Europe	North	Aarhus	AA ▲	56° 9' N	10° 13' E
	South	Barcelona	BA ▼	41° 25' N	2° 11' E
North America	North	Bellingham	BE △	48° 44' N	122° 28' W
	South	Davis	DA ▽	38° 33' N	121° 44' W

The populations used in this study and their location (see figure 2). The listed symbols are used throughout the paper.

FIGURE 2

INTERPOPULATION CROSS SCHEME



For each double headed arrow, the egg sizes and egg numbers were assessed for the population hybrid cross and the reciprocal hybrid cross. The parental phenotypes were also assessed.

interactions in egg size between males and females from different population. For example, we mated virgin BE females to AA males (BE x AA), and virgin AA females to BE males (AA x BE). In our notation, the origin of the female was always listed first and the male second. Each cross type (and the accompanying reciprocal cross) had between 20 and 30 replicates. We also crossed males and females from the same population in order to characterize the parental population phenotypes. The pairs were lightly CO_2-anesthetized and placed into a vial that contained an ice cream taster spoon with a dab of molasses-cornmeal medium and live yeast. The pairs were not anesthetized beyond the first day. Day one is the day on which the pairs were placed together. The first "food spoon" contained enough yeast and moisture to last the pair for their first two days. On the beginning of the third day, we tapped the flies to the bottom of the vial in order to replace the food spoon. This was done every 24 hours after the third day. Not all of the crosses began laying on the same day; 14% of the crosses set up in this study began laying on the third day after eclosion. Therefore the pairs that began laying on the third day had 6 days of egg size data. The pairs that began laying on the fourth day have 5 days of data. Despite the asynchrony in laying initiation, all pairs were ended at the same time, the eighth day after eclosion. On the eighth day, the flies were frozen at -80° C and desiccated overnight in an oven set at 60° C. Dry mass was obtained using a Mettler Toledo MT5 ultrabalance.

The pairs laid the F1 population hybrid eggs on the food spoon. These eggs were counted and then five eggs were haphazardly selected to be imaged. Because *D. subobscura* lays eggs into the medium rather than on the surface, the eggs had to be extracted from the medium and positioned on their side using an insect dissection pin.

Pictures were taken at 285 X magnification with an Olympus DP12 camera attached to an Olympus SZX12 dissecting scope each day for 5-6 consecutive days. If the pair laid fewer than 5 eggs, we imaged only what was available. On some days a few pairs skipped egg laying one day during the observation period.

Egg image analysis

For each replicate pair, we had five days of eggs to measure, with repeated measures on each day. We used the image analysis program NIH ImageJ to measure the projected (cross sectional) area, the major axis, and minor axis for 3 eggs of the 5 photographed eggs on each image. We calculated volume of the egg to use as our measure of egg size. Volume was calculated according to the formula for an ellipsoid:

$$V = \frac{\prod}{6} ab^2 \qquad (1)$$

where a = major axis and b = minor axis of the ellipse fitted by ImageJ.

Statistical analysis

All statistical analyses were done in the software package R version 2.2.1 (2005). We took a principal components approach to analyze the data. We computed PC scores two separate times (centered and unscaled, using the function prcomp) – once on the egg volume data and once on the egg number data. We calculated PC scores on egg volume for a 5 day observation period. Since only 14% of the cross replicates in this study began laying on day 3, we calculated the PC scores for egg size on only days 4-8 across all cross types. We also calculated PC scores on the egg number for a 6 day observation period. Even though most crosses did not start laying on the third day, their egg number

was equal to zero for day 3 and therefore this was not a missing value (unlike for egg size on day 3); therefore we were able to calculate PC scores for 6 days.

Trade-offs between progeny size and number

We used a multiple regression approach to test for a trade-off between egg size and egg number. Our multiple regression took mean egg size on post-eclosion days 6-8 as the dependent variable and had the following independent variables: female dry mass, male dry mass, population continent, population latitude, and mean egg number on post-eclosion days 6-8. We used the means of raw egg size and raw egg number because we were interested in evaluating the trade-offs in slightly more mature flies. In *D. melanogaster* during the first 20 days of egg laying, numbers initially increase rapidly and then stabilize for a few days (eventually egg number decreases with age) (Bouletreau-Merle 1971). The same is observed for *D. subobscura* (Figure 5). We therefore took our measures of egg size and number during the later period in the observations.

F2 phenotypes to assess inbreeding

We reared F1 eggs to adulthood and set up full-sib crosses to produce F2 eggs. We used these F2 eggs to assess the potential for inbreeding. If the F1 progeny make eggs that are consistently larger or greater in number, it is possible that inbreeding is present in our population lines. If inbreeding is present, interpopulation crosses may result in egg numbers that are greater than the intrapopulation crosses (Attia and Tregenza 2004), obscuring the interpretations of our results.

CHAPTER IV

RESULTS

Principal components analysis

Calculation of the PC scores for egg volume yielded 5 sets of PC scores that were independent of one another. These scores were used as our new variables. Volume PC scores were only calculated for post-eclosion days 4-8. Volume PC1 explained the greatest variance in volume among the days (74.1%) Volume PC1 was a measure of overall egg size; volumes over the five days were all positively correlated with one another. Volume PC2 explained 10.9% of the variance and was a measure of a trade-off in size between post-eclosion days 4-5 and days 6-8. A large positive volume PC2 value indicated that on days 4-5, egg size was small, while on days 6-8, egg size was large. A large negative volume PC2 value indicated that eggs were large on days 4-5 and then small on days 6-8. A zero (or near zero) volume PC2 indicated that eggs stayed the same size during the 5 days. See Table 3 for loadings of the principle components.

We also calculated PC scores for egg number over a six day period. We had 6 sets of PC scores based on egg number data but used only the first and second sets. Number PC1 accounted for 54.6% of the variance and was a measure of overall egg number; numbers on the six days were all positively correlated with one another. Number PC2 accounted for 15.5% of the variance and was a measure of a trade-off in number between post-eclosion days 3-4 and post-eclosion days 5-8. A large positive number PC2 value indicated that on days 3-4 egg number was small, while on day 5-8

TABLE 3

PRINCIPAL COMPONENT VARIANCES AND LOADINGS

Principal components analysis of egg volumes

	PC1	PC2
Standard deviation	0.119	0.046
Proportion of Variance	0.741	0.109
Loadings		
Day 4	0.3647	-0.7288
Day 5	0.4447	-0.3386
Day 6	0.4473	0.0504
Day 7	0.4908	0.3104
Day 8	0.4778	0.5054

Principal components analysis of egg number

	PC1	PC2
Standard deviation	2.5	1.33
Proportion of Variance	0.546	0.155
Loadings		
Day 3	0.0915	-0.0917
Day 4	0.7186	-0.6746
Day 5	0.4569	0.4401
Day 6	0.3203	0.2471
Day 7	0.2926	0.3292
Day 8	0.2799	0.4165

egg number was large. A large negative number PC2 value indicated the reverse. A zero (or near zero) number PC2 meant that egg number stayed the same during the observation period. See Table 3 for loadings of the principle components.

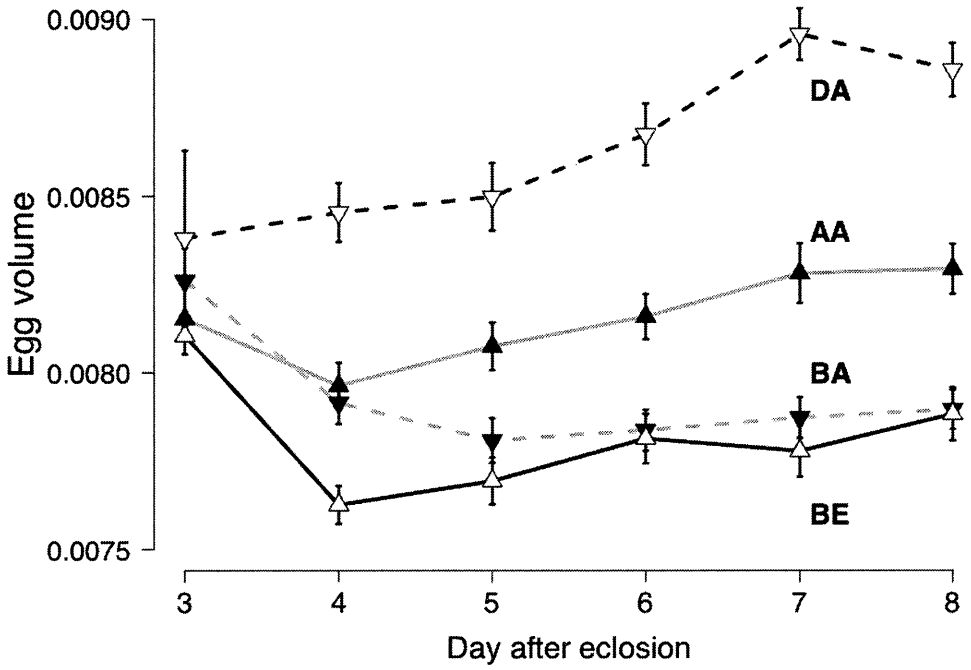
PC1 analysis for egg volume among the populations

The four populations of *D. subobscura* differed in their overall and daily patterns of raw egg volume (Figure 3). Volume PC1 (Figure 4A) among the four populations was significantly affected by latitude ($F_{1,152} = 11.34$, $P = 0.001$), and continent ($F_{1,152} = 11.74$, $P = 0.0008$). There was also a significant interaction term between latitude and continent ($F_{1,152} = 103.14$, $P < 0.0001$). In Europe, volume PC1 was larger in AA than in BA (Figure 4a). This conforms to the clinal observations made in *D. melanogaster*, whereby northern populations have larger eggs than southern populations. However in North America, the patterns of egg size did not conform to observations in *D. melanogaster*. The southern population, DA, had a larger volume PC1 value than the northern population BE. In fact, the DA eggs had the largest overall egg size of all the populations. Moreover, the BE eggs were similar in size to the BA eggs. Thus while there are egg size differences in North America, the patterns do not follow those observed in Europe, as indicated by the significant interaction between latitude and continent.

Volume PC1 was also significantly affected by female mass. Larger females laid larger eggs. Because of the significant effect of female mass on egg size, we investigated the differences in body mass among the populations. There was a significant effect of continent ($F_{1,156} = 6.36$, $P = 0.013$) and latitude ($F_{1,156} = 10.71$, $P = 0.0013$) on female mass but there was no interaction ($F_{1,156} = 0.25$, $P = 0.62$). The females from Europe were slightly larger than the females from North America. On both continents, the

FIGURE 3

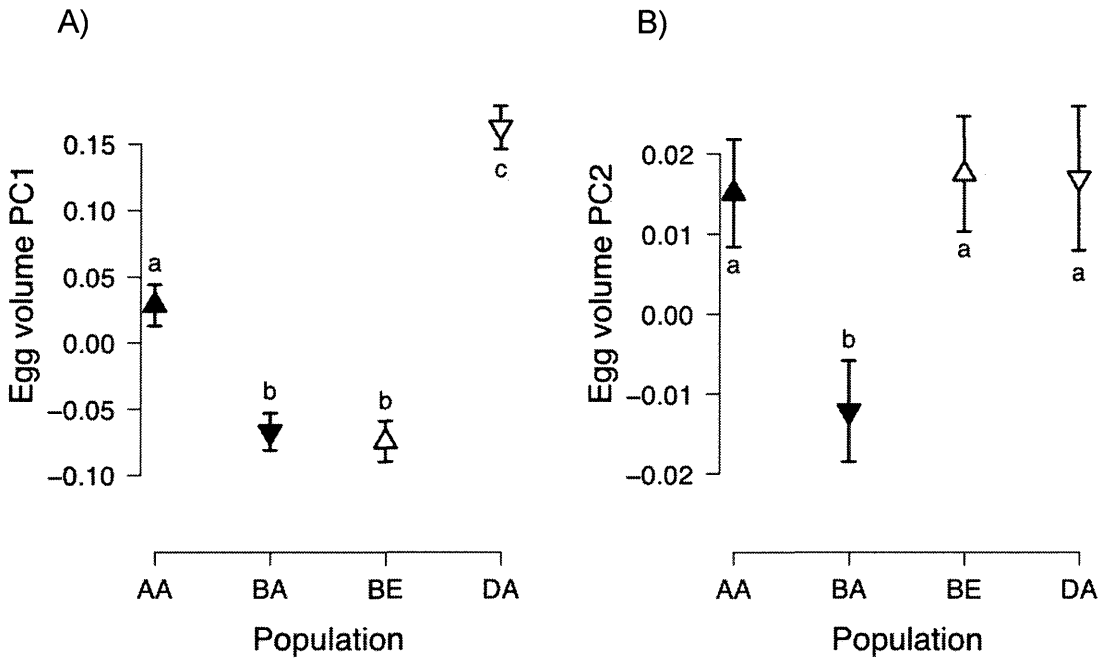
POPULATION DIFFERENCES IN EGG SIZE



The white symbols indicate the high (△) low (▽) latitude populations in North America. The black symbols indicate the high (▲) and low (▼) latitude populations in Europe. The error bars represent ±1 SE.

FIGURE 4

POPULATION DIFFERENCES IN EGG VOLUME PC1 AND PC2



A) Volume PC1 was larger in the European northern latitude population (AA) than in the southern latitude population (BA); the pattern was reversed for North America.

Different letters indicate which populations are significantly different from one another (Tukey's HSD, $p<0.05$).

B) Volume PC2 was a size trade-off between post-eclosion days 4-5 and days 6-8. Volume PC2 was AA than in BA; in North America, volume PC2 was the same in the northern (BE) and southern (DA) populations.

southern females weighed more than the northern females. Thus, interestingly, while body size was positively correlated with egg size, in Europe, the northern females were smaller but laid larger eggs; conversely the southern females were bigger but laid smaller eggs. In North America, the effects are more intuitive: the northern population flies were smaller and laid relatively small eggs; the southern flies were larger and laid bigger eggs.

The overall difference between northern and southern flies with regard to female mass is a non-intuitive result. These differences are the reverse of the clinal differences in wing size and body mass observed for *D. subobscura* populations for previous studies. Typically, northern female flies are heavier and have longer wings than southern female (Huey et al. 2000; Gilchrist et al. 2004). This discrepancy may be explained by the fact that we assayed only two populations; clines may be significant overall even if one population is an outlier. No differences in male mass among the populations were observed in this study ($F_{3,156} = 1.20$, $P = 0.31$). There was no effect of male mass on volume PC1 and it was dropped from the ANOVA.

Egg volume for individual females increased overall during the observation period (Repeated measures ANOVA, $F_{1,662} = 79.45$, $P < 0.0001$). Volume PC2 was a trade-off in egg size between early and later days and described a size change. Volume PC2 varied significantly across continents ($F_{1,152} = 4.50$, $P = 0.036$) and latitudes ($F_{1,152} = 4.83$, $P = 0.030$). In Europe, volume PC2 was larger in the northern population than in the southern population (Figure 4B). This means that in AA, eggs started out relatively small, grew slightly and changed relatively little (Figure 3); meanwhile in BA, egg size started out larger initially but then decreased and stabilized. This difference indicates that perhaps there is clinal variation for an early-late size trade-off in Europe. However, in

North America, the northern and southern populations did not vary with respect to volume PC2. Both populations had a positive PC2 value but there was not much of a size increase from days 4-8 (Figure 3). Thus, latitude has no effect in North America on volume PC2. BA turned out to be the most different population; egg volume actually decreased over time (Figure 3). AA, BE, and DA were not significantly different from one another (Tukey's HSD, $p<0.05$).

Volume PC2 correlated negatively with female mass although this effect is non-significant. However, the female mass × latitude ($F_{1,152} = 7.3$, $P = 0.0076$) interaction term was significant. This interaction can be explained by the fact that female mass differs between the high and low latitudes. There was no three way interaction.

Principal components analysis for egg number among the populations

The four populations of *D. subobscura* differed in their overall and daily patterns of absolute egg number (Figure 5) over the observation period. Number PC1 was significantly affected by female mass ($F_{1,184} = 7.11$, $P = 0.0083$), latitude ($F_{1,184} = 52.31$, $P < 0.0001$), and continent ($F_{1,184} = 5.16$, $P = 0.024$). The female mass × continent ($F_{1,184} = 21.44$, $P = 0.0413$), and latitude × continent interaction ($F_{1,184} = 52.00$, $P = 0.0016$) terms were significant. AA and BE had larger number PC1 than the southern latitude populations BA and DA (Figure 6a). The North American populations had a slightly larger number PC1 than the European populations, indicating the continent effect. The interaction between latitude and continent was due to a larger latitudinal difference in number PC1 in Europe relative to the northern-southern difference in North America. Overall, female mass was positively correlated with number PC1; larger females laid more eggs. However, it is curious that the northern female flies, which had a

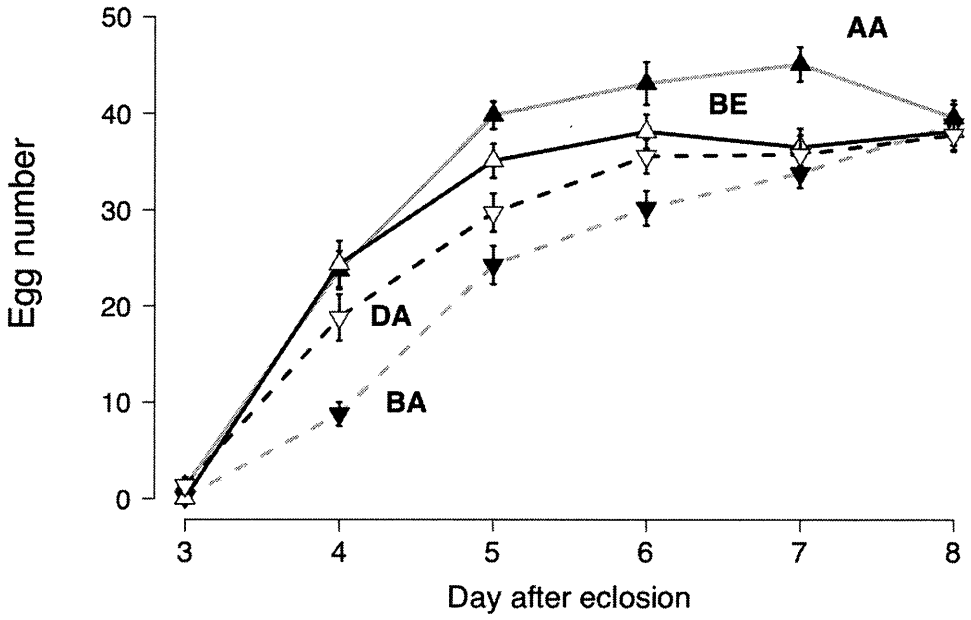
smaller body mass, actually had a larger number PC1 value. This means that the northern flies had a greater fecundity relative to their body mass, while southern flies had lower overall fecundity, relative to their larger size.

Part of the reason that the northern latitude flies had a higher fecundity was due to the fact that the populations varied in their timing of clutch initiation (Table 4). Although most pairs in this study began laying on post-eclosion day 4, 14% of the total number of pairs began laying on post-eclosion day 3. Particularly, crosses involving an AA female had the highest percentage of pairs (37.4%) that started laying eggs on day 3. About 22.5% of the total number of pairs began laying on day 5 or later (these pairs were excluded from our analyses). Crosses involving a BA female especially had the highest percentage starting oviposition on day 5 or later (30.4%). These differences may reflect fundamental life history differences between northern and southern latitude flies in the Old World. Very little difference between the northern and southern flies was observed in the New World populations. BE and DA were nearly similar with regard timing of first reproduction, and they both resemble BA. This may indicate that the North American flies have not diverged dramatically from their Mediterranean ancestors since their introduction ~25 years ago.

Egg number for individual females increased overall during the observation period (Figure 5, Repeated measures ANOVA, $F_{1,964} = 1125.7$, $P < 0.0001$). Number PC2 was a trade-off in egg number during early versus later days and described this change. Number PC2 was only significantly affected by continent ($F_{1,184} = 4.83$, $P = 0.029$). Examination of the four populations revealed that number PC2 was larger in Europe than in North America (Figure 6b). In Europe, egg number on post-eclosion days

FIGURE 5

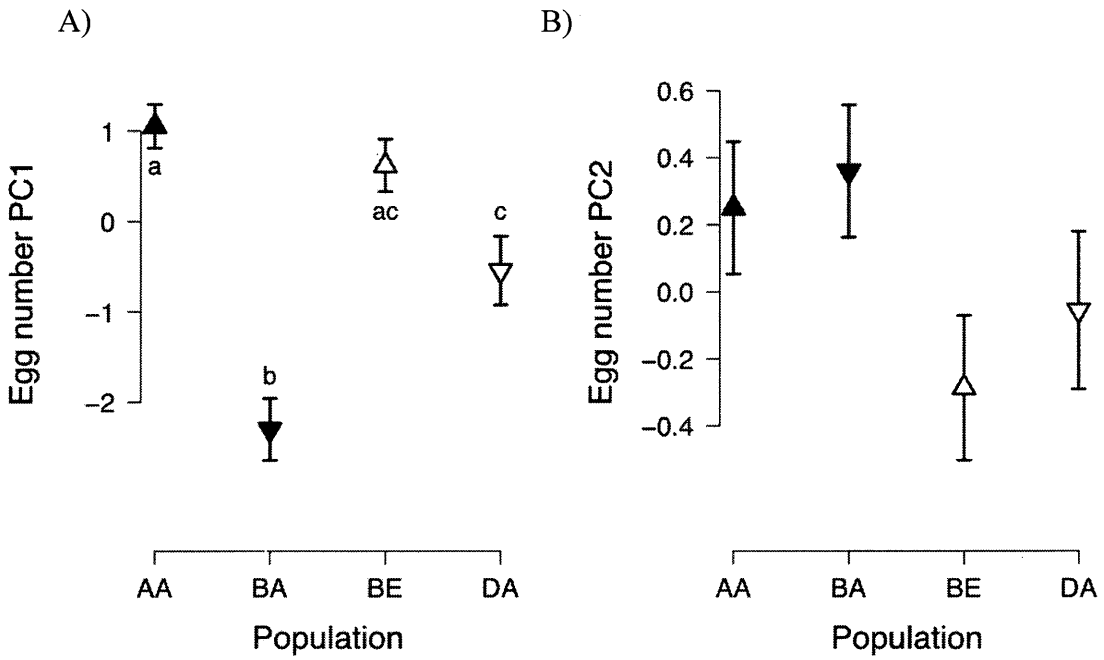
POPULATION DIFFERENCES IN EGG NUMBER



The high latitude populations had higher overall fecundity than the low latitude populations. Values represent mean ± 1 SE.

FIGURE 6

POPULATION DIFFERENCES IN EGG NUMBER PC1 AND PC2



A) The northern populations (AA & BE) have higher overall fecundity than the southern populations (BA & DA).

Different letters indicate which populations are significantly different from one another (Tukey's HSD, $p<0.05$).

B) Number PC2 is a trade-off in egg number between post-eclosion days 3-4 and days 5-8. The European populations (AA & BA) have a larger number PC2 value than the North American populations (BE & DA). The populations were not significantly different from one another.

TABLE 4

POPULATION DIFFERENCES IN CLUTCH INITIATION

	Day 3	Day 4	Day 5 or later	Total crosses
Bellingham female	9 (7.3)	83 (66.9)	32 (25.8)	124
Aarhus female	43 (37.4)	63 (54.8)	9 (7.8)	115
Davis female	7 (6.3)	75 (67.6)	29 (26.1)	111
Barcelona female	5 (4.5)	73 (65.2)	34 (30.4)	112

Listed are the percentages of when females laid for the first time during the observation period. Females were pooled across parental and hybrid cross types. Most pairs began laying on post-eclosion day 4. Percentages are in parentheses.

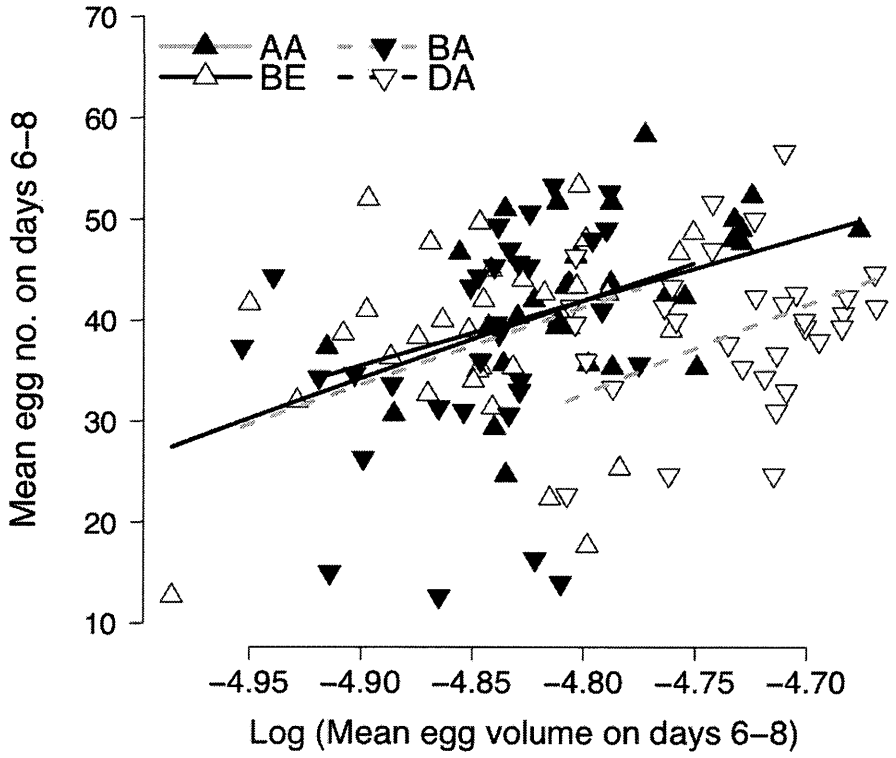
3-4 was small but increased dramatically during days 5-8. In contrast, in North America, egg number was initially larger but eventually leveled off (Figure 5). These results are curious given the North American populations are thought to have been derived from a Mediterranean population such as Barcelona (Pascual et al. *in press*). The North American flies did not resemble BA very much with respect to number PC2 (Figure 6b). In our study, latitude had no significant effect on number PC2. However, the southern populations tended to have a larger number PC2 value than the northern populations.

Trade-offs between egg size and egg number

We did not find any evidence for a trade-off between egg size and egg number in any of the four populations in this study. Rather, egg volume was positively correlated with egg number after a statistical correction for body size (Figure 7; Table 5). All the populations had the same slope and shared the same allometric relationship between size and number. However, DA differed by laying fewer eggs and larger eggs than the other three populations.

FIGURE 7

RELATIONSHIP BETWEEN EGG SIZE AND NUMBER AMONG POPULATIONS



For each population is the relationship between egg volume and egg number. There was no size-number trade-off observed in any of the four populations after a statistical correction for female body mass.

TABLE 5

ANOVA TABLE OF THE REGRESSION

	Df	M S	F	p-value
Log (Female mass)	1	330.3	4.46	0.0368 *
Log (Mean volume)	1	614.3	8.29	0.0047 *
Population	3	213.4	2.88	0.0388 *
Residuals	121	74.1		

Egg volume and egg number averaged on post-eclosion days 6-8 are positively correlated after a correction for female body size.

Principal components analysis for egg volume among the F1 hybrids

In order to study the effects of male population on egg size, we crossed a female to a male from her own population and to males from three different populations. However, to ensure that egg size did not change as an artifact of random handling differences, we tested for differences in volume PC1 in two independent trials for each of the four populations. This served as a negative control. We found no significant differences in volume PC1 in separate trials conducted. However, volume PC2 was not as robust. We did not proceed with comparing volume PC2 among the different hybrid crosses.

We found that female population ($F_{3,441} = 93.06$, $P < 0.0001$) and male population ($F_{3,441}=93.06$, $P < 0.0029$) had a significant effect on egg volume PC1. There was also a significant interaction between female and male population ($F_{9,441}=4.54$, $P < 0.0001$). Figure 8 shows how each of the females responded to the each of the male types. The panels are listed according to the latitude and the continent of the populations. Egg sizes that differ significantly from one another within a panel are labeled with different letters (Tukey's HSD $P < 0.05$). Egg size of the female increased, decreased, or remained the same in hybrid crosses relative to the parental crosses. In general, there was a strong maternal influence on egg size. This was especially true whenever the female in the hybrid cross had genetically small eggs. For example, BA and BE mothers laid the same size eggs in hybrid and parental crosses. However, there also were prominent male effects on egg size. These were seen most clearly when the female had genetically large eggs (AA and DA mothers) and was mated to a male with the genes for small eggs (BA and BE fathers). Egg size decreased relative to the parental cross in AA x BA, DA x BE,

and DA x BA crosses. In one instance, egg size actually increased relative to the parental cross (AA x DA).

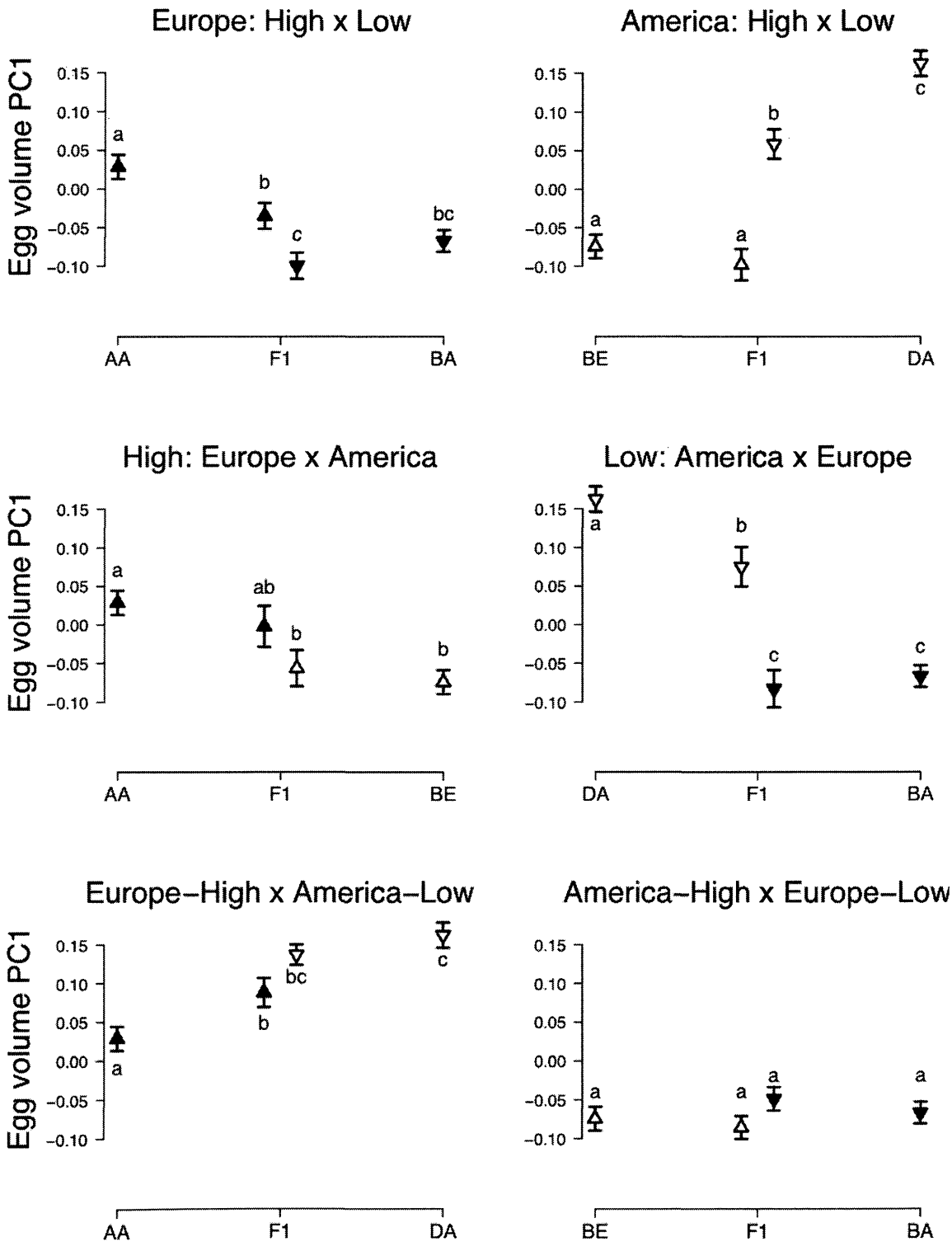
The interaction effect of male and female population on egg size can be explained by the fact that each female had a different response to a particular male. The hybrid eggs could vary with respect to each another – even though the hybrid eggs did not vary with respect to the parental cross eggs. For example, the AA x BA eggs were smaller than the AA x DA eggs. Yet, neither of these crosses was significantly different from the parental cross AA x AA (Tukey's HSD, $P < 0.05$). Also, as observed earlier, there was a significant effect of female mass on egg size; larger females laid larger eggs ($F_{1,441} = 28.92$, $P < 0.0001$).

FIGURE 8

EFFECTS OF FEMALE AND MALE POPULATION ON EGG VOLUME PC1

Individuals used in the interpopulation crosses are listed on the right and left sides of the x-axis. The reciprocal F1 progeny are in the center. The symbols represent the population of the female used in the cross.

Strong maternal effects on egg size were present. Generally, interpopulation males decreased egg size when the females had genetically large eggs. Different letters indicate which egg sizes are significantly different within each panel (Tukey's HSD, $p<0.05$).


Europe: High x Low
Egg volume PC1
0.15
0.10
0.05
0.00
−0.05
−0.10
a
b
c
bc
AA
F1
BA
America: High x Low
a
a
b
c
BE
F1
DA
High: Europe x America
a
ab
b
b
AA
F1
BE
Low: America x Europe
a
b
c
c
DA
F1
BA
Europe−High x America−Low
a
b
bc
c
AA
F1
DA
America−High x Europe−Low
a
a
a
a
BE
F1
BA

Principal components analysis for egg number among the F1 hybrids

We first tested to see if number PC1 and number PC2 were affected by handling differences during two independent trials of the same cross. We found that number PC1 and PC2 did not differ significantly between trials. This enabled us to test for the effects of male population on egg number.

Female population ($F_{1,525} = 77.32$, $P < 0.0001$) and male population ($F_{1,525} = 3.03$, $P = 0.029$) affected number PC1 significantly. There was also a significant female population × male population interaction ($F_{1,525} = 2.21$, $P = 0.020$). Figure 9 shows these results. Egg numbers that differed significantly from one another within a panel are labeled with different letters (Tukey's HSD $P < 0.05$). It is clear that females laid the same overall number of eggs, regardless of the origin of the interpopulation male. However, there was a male effect and the female × male interaction effect ($F_{9,525} = 3.70$, $P = 0.012$) on egg number. As observed earlier, female mass ($F_{9,525} = 45.21$, $P < 0.0001$) had a significant effect on number PC1.

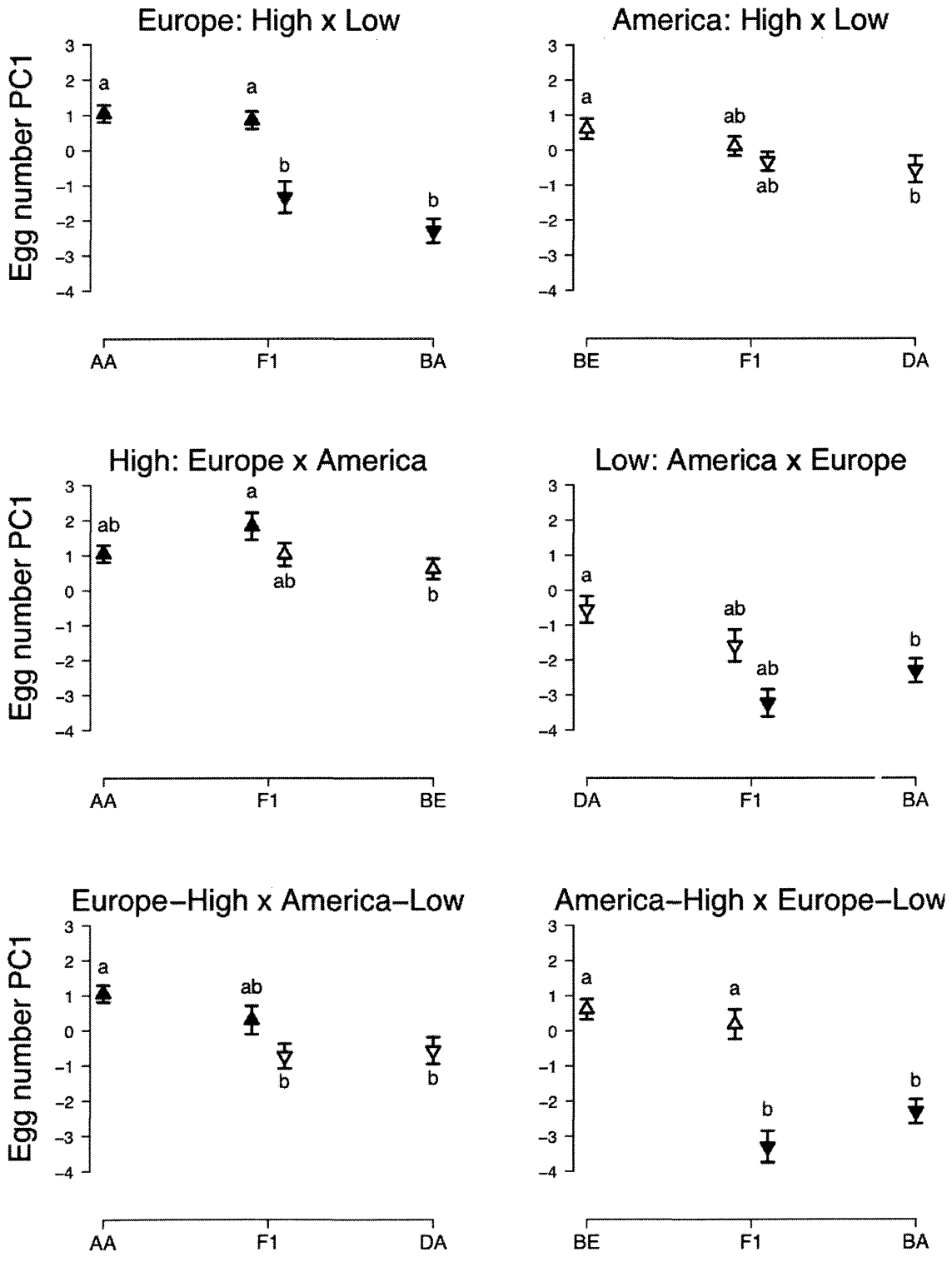
Female population affected number PC2 significantly ($F_{3,525} = 4.44$, $P = 0.0043$), suggesting strong maternal effects for the trade-off in egg number among days. In general, change in egg number over time was not generally dependent on the male population ($F_{3,525} = 1.10$, $P = 0.34$). In individual comparisons, there was only one instance of a male population effect on number PC2; the BA x BE cross resulted in a PC2 value significantly different from the BA x BA cross. The male × female population interaction was significant ($F_{9,525} = 3.09$, $P = 0.0012$). This was due to the fact that each female type responded differently to each male type. There was also a three way interaction between female mass, male, and female population ($F_{3,525} = 2.15$, $P = 0.024$).

FIGURE 9

EFFECTS OF FEMALE AND MALE POPULATION ON EGG NUMBER PC1

Individuals used in the interpopulation crosses are listed on the right and left sides of the x-axis. The reciprocal F1 progeny are in the center. The symbols represent the population of the female used in the cross.

Egg number was primarily determined by the female. Different letters indicate which populations are significantly different from one another within a panel (Tukey's HSD, $p<0.05$).


Europe: High x Low
Egg number PC1
3
2
1
0
−1
−2
−3
−4
a
a
b
b
AA
F1
BA
America: High x Low
a
ab
ab
b
BE
F1
DA
High: Europe x America
ab
a
ab
b
AA
F1
BE
Low: America x Europe
a
ab
ab
b
DA
F1
BA
Europe−High x America−Low
a
ab
b
b
AA
F1
DA
America−High x Europe−Low
a
a
b
b
BE
F1
BA

F2 phenotypes to assess inbreeding

In order to assess potential problems with inbreeding in our experiments, we counted and measured eggs laid by the F1 hybrids. We did not find that the F2 eggs varied significantly in size from the parental generation. F1 hybrids did not lay consistently larger eggs than the average egg size of their parents. This leads us to conclude that inbreeding within parental populations does not explain why egg size increased once in a hybrid cross. However, the F1 hybrids did lay more eggs than their parents (unpublished data), suggesting that the parental populations were slightly inbred. However, egg number was under the influence of the female in the interpopulation crosses; females did not change the number of eggs laid based on the male. We can rule out the hypothesis that females laid more eggs with interpopulation males because these males were perceived as genetically different.

The influence of F_{ST} on female response.

For all the interpopulation crosses, we found a strong maternal influence on egg size. Occasionally, there were male effects on egg size, but these effects were not dependent on the level of genetic differentiation between the two populations being crossed. If the strength of the change in egg size were dependent on F_{ST} values, then the smallest egg size changes should occur for crosses between individuals from populations that have low F_{ST} values. For example, F_{ST} was the lowest between populations within continent (Table 2). However, interpopulation crosses within continents could result in strong egg size changes (i.e. DA x BE and AA x BA cross) or no changes at all (i.e. BA x AA and BE x DA). Conversely, the highest F_{ST} was between populations of different latitudes and continents. Hybrid crosses between these populations resulted in no male

effects (i.e. reciprocal BE x BA crosses) or have strong male effects (i.e. DA x BA). The amount of genetic differentiation was not correlated to the degree of egg size change. This may be because measures of F_{ST} based on neutral markers are inappropriate to use when looking at mating interactions, which may involve loci that are under greater selection pressure (i.e. Acp loci show strong positive selection). In general, when egg size did change, only the original size of the egg seemed to matter. In general, females that laid genetically large eggs had stronger responses to interpopulation males than did females that laid genetically small eggs.

Maternal effects on egg number were quite strong. Although there were paternal effects on egg number, females did not generally a different number of eggs depending on the male to whom she was mated (Figure 9). This may indicate that F_{ST} values for the populations of *D. subobscura* used in this study were too low to observe male effects on egg number. The Acps that control oviposition rate may not have diverged significantly among the continental populations despite a ~25 year long separation. Alternatively, strong maternal effects on egg number may indicate that males have no effect on oviposition rates in this species.

CHAPTER V

DISCUSSION

Volume PC1 differences in the parental populations

The differences in overall egg size (volume PC1) among the high and low latitude *D. subobscura* populations did not consistently conform to the differences observed in *D. melanogaster*. In Europe, the northern latitude population, AA, had larger eggs than the low latitude population. This is consistent with observations in *D. melanogaster* (Azevedo et al. 1996). Larger eggs may be the result of an increased vitellogenesis rate relative to the oogenesis rate at cooler temperatures (Ernsting and Isaaks 1997). However, larger eggs may also be adaptive because they have higher survivability at colder temperatures than small eggs (Yampolsky and Scheiner 1996; Fischer et al. 2003). This may lead to the selection of larger eggs in higher latitude populations. Selection may have also differentiated with respect to the genes for egg size, so that relatively large eggs are observed regardless of the ovipositing temperature. This explains why AA and BA still had noticeably different egg sizes even though they were reared at the same temperature for 2 years prior to this study.

However, in North America, the high latitude population, BE had smaller eggs than the low latitude population, DA. Furthermore, DA egg size exceeded that of all the other populations, while BE egg size was very similar to the European southern population BA. This may have been the result of selection forces other than temperature operating in the New World. Low latitude sites are more arid than the high latitude sites

(NOAA 1994). This is especially true of Davis, California, which is further inland than Barcelona, Spain. If the DA population experienced a more arid environment, it is possible that larger egg size is the result of selection for desiccation tolerance. Work on mosquito and butterfly eggs has shown that large eggs have higher survivability than small eggs in conditions of low relative humidity (Sota and Mogi 1992; Fischer et al. 2005), presumably because of the lower surface area to volume ratio. Furthermore, it is known that *D. subobscura* is less common at lower latitudes in the New World (Noor 1994). Flies that persist at lower latitudes may be under strong selection for desiccation resistance – leading to the evolution of larger eggs. In general, larger body size is a potential adaptation for desiccation resistance.

Consistent with the interpretation that desiccation resistance may have selected for large eggs in DA is the finding that BE has relatively small eggs, which are similar in size to BA eggs. BE is located in Bellingham, Washington which receives more precipitation in the spring (NOAA 1994). North American populations of *D. subobscura* flourish particularly in these more humid high latitude environments; it has long been the dominant obscura-group species in these areas, displacing the native obscura-group species *D. pseudoobscura* (Pascual et al. 1998). Nonetheless it is peculiar that egg size has not evolved larger eggs in the cool environment of Bellingham, which experiences temperatures that are equivalent to those in Aarhus during the spring breeding season. The reason for this discrepancy may also explain why the cold temperatures do not always result in larger eggs in other organisms (Kussano and Kussano 1988; Baur and Raband 1988; Fleming and Gross 1990).

Larger eggs in Davis, CA may also occur because of competition; smaller eggs in Bellingham, WA may occur because the competition is mitigated. Parker and Begon's (1986) model of optimal egg size predicts that larger egg size should be favored under conditions of high sibling and non-sibling competition. Field observations from southern California reported that *D. subobscura* flies coexist with other obscura-group flies but are relatively scarce. In Mather, CA (37° 57' N), which is 30 miles west of Davis, CA, *D. subobscura* accounted for only 1% of the proportion of species collected (Noor et al. 1998). Other obscura-group flies, such as *D. pseudoobscura*, *D. persimilis*, and *D. azteca*, had higher relative abundances at these latitudes. *D. subobscura* population sizes vary throughout the year, and seasonal abundance patterns are similar to that of the endemic obscura-group species in Davis, CA (Pascual et al. 1993). If *D. subobscura* has to share breeding sites with other obscura-group flies, strong competition may select for larger egg size. Additional studies have shown that *D. subobscura* was out competed in laboratory situations with *D. pseudoobscura*, which is more fecund and has a higher female-biased sex ratio in laboratory conditions (Pascual et al. 1998; Pascual et al. 2004), suggesting that at least one endemic species is more competitive under certain conditions than the invasive *D. subobscura*. Competition may favor larger egg size in the DA population because larger eggs have higher larval fitness (Azevedo et al. 1997).

The relative small size of BE eggs is consistent with this hypothesis. Since *D. subobscura* is the now dominant species in the higher latitude sites of North America (Noor et al. 1998), selection for increased egg size is probably much abated. In Europe, the situation is reversed (reviewed in Krimbas 1993). *D. subobscura* is the dominant species at low latitude locations like Barcelona, Spain. On the other hand in central and

northern Europe, *D. subobscura* is less common and coexists with other obscura-group species such as *D. helvetica*, *D. subsilvestris*, *D. alpina*, and *D. obscura*. If competition has a large effect on egg size, one would predict that AA eggs are large while BA eggs are relatively smaller. While *D. subobscura* competes with other *Drosophila* species for oviposition substrates such as rotting fruit (Atkinson 1979), the native oviposition substrates of *D. subobscura* are poorly known. Rotting fruit may not be the primary breeding and larval sites. If we are to explain why *D. subobscura* abundances vary, there must be further investigation into the breeding ecology of this species in relation to its competitors.

Volume PC2 differences in the parental populations

The trade-off in egg volume on days 4-5 versus days 6-8 yielded a peculiar difference among the continental populations. The European northern population (AA) had a larger volume PC2 value than the southern latitude population (BA). This means that AA egg size started smaller, increased slightly, and then stabilized; BA egg size started out large and then decreased. This result is correlated with the differences in timing of egg laying. Early on, an AA female's eggs may spend a shorter amount of time in vitellogenesis (relative to eggs produced later on) if she begins oviposition earlier. In other words, initiation of egg laying may correlate positively with egg size. On the other hand, the BA flies, initiated laying later on average, perhaps indicating that they mature slightly more slowly. If BA females take longer to lay eggs but are all the while undergoing oogenesis and ovulation, their eggs may be initially larger. Their first eggs may spend more time in vitellogenesis and thus be larger – leading to an early-late trade-off in size.

By contrast, a difference in volume PC2 (trade-off between post-eclosion days 4-5 and days 6-8) between the northern latitude (BE) and southern latitude (DA) populations did not occur in North America. This is interesting for two reasons. Firstly, BE and DA populations differ markedly in overall egg size (volume PC1). One might have expected that they would also demonstrate a difference in early versus later egg size trade-off, as in Europe, but no difference is manifest. Secondly, the New World *D. subobscura* populations are likely derived from the Mediterranean region in Europe. If this is the case, we would expect that at least the southern population in North America, DA, to resemble BA in terms of volume PC2. However, neither DA or BE resemble BA; rather, they resemble AA in this regard.

Number PC1 differences in the parental populations

Overall egg number differences between high and low latitude populations on both continents were similar. Northern flies produced more eggs during the observation period than the southern flies. This effect is partially driven by the earlier initiation of oviposition in higher versus lower latitude flies. These observations are consistent with work in *D. melanogaster*, which exhibits clinal variation in development time. When populations collected at different latitudes are reared in the lab at the same temperature, development time is inversely correlated to latitude (James and Partridge 1995). If egg-to-adult development time is a proxy for reproductive maturation, we would expect northern latitude flies to initiate egg laying sooner than southern latitude flies. We observed such a difference in Europe between AA and BA. However, the New World *D. subobscura* northern and southern populations had only a minor difference in the percentage of day 3 initiation. The New World populations in higher and lower latitudes

may not have diverged in development time. This is an unusual result as laboratory selection experiments have shown that populations reared at low temperatures obtain faster development times within 5 years when compared to high temperature lines (Huey et al. 1991; Partridge et al. 1994; James and Partridge 1995). One might expect that the New World *D. subobscura* populations, which have been present for ~25 years, would have evolved clinal differences in development and maturation rates in that length of time. If differences in development time between high and low latitude populations have not occurred, this is again suggestive that environmental factors other than temperature have influenced the evolution of *D. subobscura* in at least North America. We suggest assays in development time among a latitudinal cline to confirm our observations.

Number PC2 differences in the parental populations

Number PC2 (the trade-off in number between post-eclosion days 3-4 and days 5-8) did not vary significantly among the populations. The southern populations had a slightly larger number PC2 value than the northern populations; this may be indicative of maturation differences in southern versus northern flies as discussed earlier. If northern flies develop faster than southern flies (when reared at the same temperature), they may initiate egg laying sooner as observed in this study. However, they may only be able to make relatively few eggs at first and may still take a little while to ramp up oogenesis. However, the difference between latitudes was not significant.

There was a significant effect of continent on number PC2; the European populations had a larger number PC2 value than the North American populations. This means that the European flies increase egg number over time, while the North American flies remained the same or decreased slightly. Again, it is peculiar that the New World

populations do not resemble BA more since the New World populations are presumed to be from the Mediterranean region of Europe. So it seems that the New World populations have diverged from, rather than converged upon the Old World patterns with respect to number PC2, as was the case with volume PC2. The reason for this divergence is unclear and requires more investigation.

Trade-offs between egg size and egg number

We did not observe any trade-offs between progeny size and number in any of the four populations, leading us to conclude that trade-offs may not occur under lab rearing conditions especially since the assumption of fixed resources was not satisfied in our study (Smith and Fretwell 1974). Fischer et al. (2005) only found weak evidence for a size-number trade-off in butterfly eggs when individuals are provided with food *ad libitum*. They discovered that females increased egg size by increasing water content and fresh mass; however, these females also experienced a steep decline in egg size with age. This is one example where trade-offs were examined in an environment where food is unlimited. Van Noordwijk and de Jong (1986) investigated positive correlations in life history traits by examining the relative genetic variation for acquisition and allocation ability. Their model demonstrated that when the genetic variation in resource allocation (to different components of life history) is large relative to variation in resource acquisition, there will be a negative correlation between the components under study. In other words, if food is scarce, allocating resources to some aspects of life history relative to others becomes a pressing issue for all individuals and trade-offs occur. On the other hand, if allocation variation is small relative to acquisition variation, positive correlations arise. When food is abundant, no trade-offs occur. In Fischer et al.'s (2005) study and in

our study, the variation in allocation of resources into egg size versus egg number may have been smaller than the variation in food acquisition ability in egg production. Therefore, there was a positive correlation between egg number and size. A simple way to increase variation in allocation ability (relative to acquisition ability) would be to limit food.

Male-effect on volume PC1

We observed that males were able to influence the overall size (volume PC1) of their progeny. Egg size increased, decreased, or remained the same when a female was crossed with an interpopulation male. Each female also responded differently with the same interpopulation male. This is a novel result for an oviparous insect species like *D. subobscura*. In *D. subobscura*, eggs are fertilized just prior to oviposition and are not retained for a significant period of time. This would seemingly suggest that there is little opportunity for the male or the progeny to obtain more nutrients (for the egg) from the female. However, male sperm have the opportunity to fertilize two kinds of eggs: eggs that are fully formed before copulation and eggs that are formed after copulation. We do not expect that males can manipulate egg size of the mature eggs that virgin females retain prior to copulation; but suggest that males can affect the size of the eggs that are formed after mating. Because of the repeated measurements over a 5 day observation period, we are sure most the eggs are fertilized.

At the present, it is unclear how males are capable of affecting the size of their progeny. Weigensberg et al. (1998) found a paternal genetic effect on egg size in the cricket *Gryllus firmus*. *G. firmus* eggs take several days to mature and during this time they undergo size changes due to metabolism and water uptake. Females mated to wild-

type and mutant males laid eggs of similar size, but ten days later they observed that eggs of mutant males were smaller. While *D. subobscura* eggs do grow slightly over a ~12 hour period (eggs begin hatching ~12 hours after being laid), the size change is still an order of magnitude smaller than the size change that occurs in hybrid crosses relative to parental crosses (unpublished results). It is therefore not possible for the *D. subobscura* father to affect egg growth and metabolism appreciably. If growth effects are present, they may be more apparent only in larvae. Rather, the male-effect on size must be due to either a behavioral or a biochemical interaction.

Males may affect the egg size of their female partners via Acp molecules. Although no Acps have been identified that directly control the rate or duration of vitellogenesis, there is some suggestion that this is not a far-fetched prediction. A recent study has shown that Acps enter into and bind to targets in the female ovaries. In addition, some Acps are embedded in the egg shell (Ram and Wolfner 2005). Finally, it is well known that Acps can affect oogenesis, ovulation, and oviposition rates (Chen et al. 1988; Heifetz et al. 2000; Saudan et al. 2002). If male Acps stimulate females to lay many eggs, this may limit the amount of time an oocyte spends in vitellogenesis (i.e. a size-number trade-off). While fertilizing many eggs is desirable, the male may also profit by investing in quality instead of quantity, given the fitness benefits of increased egg size.

However, given that we observed that egg size from interpopulation crosses decreased 3 out of 4 times relative to the parental population egg size (Figure 8), it is also possible that reproductive incompatibilities have arisen among populations for which this effect was observed. Kondoh and Higashi (2000) concluded from theoretical modeling that mismatches between male growth promoters and female growth suppressors

expressed in progeny may result in postzygotic isolation due developmental problems in the progeny. In *Drosophila*, this may be akin to mismatches in female receptors for male Acps that affect egg size.

Interpopulation males may harass females more or less (or the same) than intrapopulation males; different rates of behavioral harassment may lead to increased or decreased rates of energy expenditure that shunt resources away from or into vitellogenesis. Alternatively, females may respond differently to the courtship behaviors by interpopulation males by altering their investment decisions. Females may make larger eggs when presented with more attractive males. In some birds it is clear that females have higher reproductive success with attractive males compared to unattractive males. Increased reproductive success can occur through increased allocation of resources into eggs, resulting in larger eggs (Cunningham and Russell 2000), or through increased fledging success (Swaddle 1996). Although the differential allocation hypothesis (Burley 1986) has been studied in insects, no one has yet manipulated attractiveness in insects and looked for a correlated response.

Previous work in *D. melanogaster* (Azevedo et al. 1997) did not find an effect of male population on egg size. The discrepancy may partially be explained by differences in methods. Azevedo et al. (1997) measured eggs from flies aged 5-7 days for a 25° C treatment (or 9-11 days for the 18° C treatment); our study measured eggs from the day of first day of egg laying until the flies were 8 days old. We were able to consider the overall egg size of individual females by taking repeated measurements. Because egg size is sensitive to environmental conditions experienced by females, taking repeated measurements helps to account for this random variation and may have made it more

likely to detect differences in egg size. Also, we achieved higher precision in our size measurements by taking pictures of the eggs at a higher magnification. Although the male-effect on egg size is small compared to the female-effect, our results show clearly that males can play a role.

We found no effect of the degree of population differentiation on the degree of the female response to an interpopulation male in volume PC1. The only consistent result was females that laid large eggs had a different egg size whenever mated to a male with the small egg genotype, suggesting that perhaps genetic differentiation has no effect on egg size changes. Alternatively, measures of F_{ST} based on microsatellites may not be as good as measures based on loci related to mating (i.e. Acp genes). Our results are perhaps indicative of male-female mismatches in either behavioral or hormonal cues that cause changes in progeny size. To test this theory more adequately, future studies should score interpopulation progeny for fitness characteristics. If hybrid progeny have lower fitness relative to the parental fitness, changes in egg size in interpopulation crosses may be maladaptive.

Male-effect on number PC1

As seen in other studies, we found significant effects of female population, male population, as well as an interaction between the two on the number of eggs laid by a female. These significant results may be indicative of a variety of processes of sexual selection including sexual conflict and female choice. We found a particularly strong effect of female population on overall egg number. Females did not have consistently stronger or weaker responses of males from different populations. This may indicate that the continental populations did not diverge enough to result in differences between the

male Acp molecules and female receptors involved in oviposition. This is a somewhat surprising result given that other interpopulation cross studies have shown male effects on oviposition rates.

The question of interpopulation crosses

Although Rowe et al. (2003) concluded that interpopulation crosses cannot by themselves diagnose the process of sexual conflict, we find that the interpopulation cross technique ought not to be wholly abandoned. Our discovery of male effects on insect egg size is a perfect example of the continued utility of interpopulation crosses in studies of male-female coevolution. Male effects on egg size were not suspected for an organism like *Drosophila*, since females do not retain fertilized eggs for a significant period. Indeed, this is the first study in any insect species where a female has been found to alter egg size depending on the male to which she has been mated. The presence of this effect in insects, already found in birds, justifies further study into the mating interactions affecting insect egg size. Future researchers could investigate avenues of sexual conflict over egg size or even differential allocation in terms of egg size in *Drosophila*. Follow up studies should also be targeted at discovering how these males are able to produce the effects that we have observed. Is altered egg size behaviorally or hormonally induced? Is it a seductive or a manipulative effect? Interpopulation crosses were successful in revealing the presence of this effect, indicating that this technique can still contribute to our knowledge of sexual conflict or female choice, at least by revealing unsuspected mating interactions.

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